CHARACTERIZATION OF GSTA3 GENE PRODUCTS IN MULTIPLE SPECIES AND DEMONSTRATION OF THEIR CONSERVATION IN DIVERGENT SPECIES

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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May 2015

Major Subject: Animal Science

ABSTRACT

The presence and activity of glutathione S-transferase alpha 3 (GSTA3) in the testosterone biosynthesis pathway of humans and multiple mammalian species is a new concept. Some researchers have hypothesized that the gene encoding GSTA3 is conserved across many other species. This study undertakes characterization of the GSTA3 gene products in multiple diverse species in order to demonstrate its conservation. The species analyzed here include *Canis lupus familiaris*, *Capra hircus*, *Monodelphis domestica,* and *Gallus gallus*. Testis samples from each of these species were taken, RNA was isolated, reverse transcribed, and the GSTA3 cDNA was cloned and sequenced with polymerase chain reaction (PCR) technologies. cDNA sequences were then analyzed with the NCBI BLAST algorithm, ExPASy Translate tool, and Clustal Omega Multiple Sequence Alignment tool. These tools were also used to compare the cloned cDNAs to a reference sequence from *Homo sapiens* (GenBank accession number XM_000847.4) and an *Equus caballus* sequence published in the NCBI database (KC512384). GSTA3 cDNA sequences obtained for each species were submitted to NCBI GenBank. A set of multiple tissue samples was taken from both *Equus caballus* and *Canis lupus familiaris*. These tissue samples were analyzed for the expression of GSTA3 mRNA in each tissue after RNA isolation, reverse transcription and quantitative polymerase chain reaction (qRT-PCR). cDNA data analysis demonstrated GSTA3 gene conservation at the DNA level in all the species studied.

Real time quantitative PCR data demonstrated significantly high concentrations of the GSTA3 mRNA in the testes and adrenal glands as compared to all other tissues for the equine multi-tissue analysis as expected. Dissimilarly, the canine multiple tissue analysis indicated that the concentration of GSTA3 mRNA was high in the liver and small intestine as well as the testes and adrenal glands. Other researchers have reported high concentrations of GSTA3 mRNA in the liver and small intestine previously.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Nancy Ing for providing me with this opportunity. Dr. Ing, your patience and willingness to help me learn and excel in the laboratory has not only been educational, but also inspiring. The countless hours you have spent teaching me and showing me the excitement of reproductive physiology have opened up a career path I hadn't previously considered and in doing so helped me discover a new dream.

I would also like to thank my committee members, Dr. Clare Gill and Dr. Martha Vogelsang, for their unfaltering support and understanding. Dr. Vogelsang, you are a wonderful professor and I have learned so much from you over the last two years. You have also allowed me to help out in the foaling class and thus provided amazing experiences, as well as your wonderful guidance. Dr. Gill, I am grateful for your willingness to be a part of this project even though it was already nearing its completion, and for all of the knowledge and support which you have imparted to me throughout this time.

Dr. Clay Cavinder, although not a part of my committee due to accepting a position at another university, was instrumental in this achievement and I am extremely grateful for the opportunity which he granted me by accepting me into this program. My experiences at Texas A&M University have not only enlightened me, but also enriched my life. Dr. Cavinder, you have helped me to open new doors to my future and for that I

will always be grateful. Your guidance was invaluable in my search for direction and purpose for my education.

Thanks also go to my friends and colleagues, Colleen O'Reilly, Raul Valdez, Jennifer Zoller, Caitlin Vonderohe, Ari Edgmon, Courtney Phillips, Meredith Kahn, and Amanda Bradbery. You all have been great friends, but you have also been so encouraging and supportive throughout this process, not to mention the numerous questions and issues we have all helped each other through over the last two years.

Finally, I want to thank my family and those I consider family, and dedicate this achievement to them. Without all of your emotional and financial support I could not have completed this degree. The Lord has truly blessed me by bringing all of you into my life. I hope only that you all know how much I love each and every one of you, and how grateful I am for everything you do for me.

NOMENCLATURE

11B-HSD1	11-beta hydroxysteroid dehydrogenase type 1
11B-HSD2	11-beta hydroxysteroid dehydrogenase type 2
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
ChIP	Chromatin Immunoprecipitation Assay
GSH	Glutathione
GST	Glutathione S-transferase
GSTA3	Glutathione S-transferase Alpha 3
GSTP	Glutathione S-transferase Pi
GSTM	Glutathione S-transferase Mu
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta-steroid delta-isomerase 2
HSD17B3	hydroxysteroid (17-beta) dehydrogenase 3
NC	Negative Control
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
qRT-PCR	Real Time Quantitative Polymerase Chain Reaction
SF-1	Steroidogenic factor 1
SNP	Single Nucleotide Polymorphism
StAR	Steroid Acute Regulatory Protein

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Although the testosterone biosynthesis pathway is well studied there are still gaps in our knowledge and understanding of its mechanisms (Agular et al., 1992; Badrinarayanan et al., 2006; Board, 1998; Diemer et al., 2003; Gu et al., 2004; Gunasekar et al., 1988; Herrera-Luna et al., 2012, 2013; Ing, 2005; Johansson and Mannervik, 2001, 2002; Kanzaki and Morris, 1999; Laughlin et al., 2010; Namiki et al., 1991; Payne and Hales, 2004; Payne and Youngblood, 1995; Tars et al., 2010; Yoon and Roser, 2011). Testosterone is produced by the Leydig cells of the testis from cholesterol through the actions of a series of enzymes. Species specificity has been demonstrated in the steroidogenic pathways, with rodent species utilizing the Δ4 pathway and human and livestock species utilizing the Δ5 pathway (Johansson and Mannervik, 2001, 2002; Larsson et al., 2011). It has been discovered that human and livestock species employ the enzyme glutathione S-transferase alpha 3 (GSTA3) as a steroid isomerase to convert △5-androstenedione to △4-androstenedione, the immediate precursor of testosterone (Calvaresi et al., 2012; Dourado et al., 2014; Gu et al., 2004; Hayes et al., 2005; Johansson and Mannervik, 2001, 2002; Matsumura et al., 2013; Raffalli-Mathieu et al., 2008). Recombinant human GSTA3 has been demonstrated to be 260 times more efficient in isomerizing Δ5-androstenedione to Δ4-androstenedione than HSD3B2, the enzyme to which this activity was previously attributed (Dourado et al., 2014; Johansson

and Mannervik, 2001, 2002; Larsson et al., 2011; Matsumura et al., 2013; Morel et al., 2002).

There is little information related to the regulation of the GSTA3 gene and its actions in steroidogenesis. This is in part due to the fact that the GSTA3 gene is only highly expressed in steroidogenic tissues. In addition, GSTA3 is a member of a large family of highly related GSTA genes clustered on one chromosome. The gene products are difficult to distinguish from one another, and they are all also expressed in Leydig cells as well as other tissues (Board, 1998; Dourado et al., 2014; Johansson and Mannervik, 2001, 2002; Larsson et al., 2011; Luu-The, 2013; Morel et al., 2002). Further research to elucidate which species utilize GSTA3 is of great importance. Evidence has been provided that a synthetic glucocorticoid, similar to natural stress hormones, has a suppressive effect on the expression of the GSTA3 enzyme in stallion testes (Ing et al., 2015). Considering most of our food production animals are housed in confinement type operations which are generally very stressful environments, it is necessary to understand the regulation of the GSTA3 gene and the role of the GSTA3 enzyme in testosterone biosynthesis. To this aim, many researchers have utilized the corticosteroid drug dexamethasone to mimic stress in research animals in order to study its effects on testosterone production. A few researchers have focused specifically on the effects of glucocorticoids like dexamethasone and its effects on steroidogenesis (Badrinarayanan et al., 2006; Barth and Bowman, 1994; Danek, 2004; Hardy et al.,

2005; Ing et al., 2014, 2015; Wang and Stocco, 2005; Xiao et al., 2010). However, few studies have focused on determining the evolutionary conservation of the GSTA3 gene across species. The following project will examine the GSTA3 mRNA expression in the testis and other body tissues, as well as the specific coding sequence and sequence conservation between multiple species, which will include *Equus caballus, Canis lupus familiars* and *Capra hircus*, as well as divergent species*, Monodelphis domestica* and *Gallus gallus*. These data will provide a more thorough understanding of the GSTA3 gene products and their regulation of expression.

The purpose of this study is to:

provide further evidence that the GSTA3 gene is most highly expressed in the gonads and adrenal glands, as well as elucidate and compare the GSTA3 cDNA sequence across species such as *Homo sapiens, Equus caballus, Canis lupus familiaris, Capra hircus*, *Monodelphis domestica, Gallus gallus*.

CHAPTER II

LITERATURE REVIEW

Steroidogenesis

Physiological, pathological and developmental processes are brought about by the actions of steroid hormones. These hormones consist of estrogens, androgens, progestogens, mineralocorticoids and glucocorticoids (Almadhidi et al., 1995; Carreau et al., 2001; Conley and Bird, 1997; Evain et al., 1976; Gummow et al., 2006; Ing, 2005; Johansson and Mannervik, 2001; Kanzaki and Morris, 1999; King and LaVoie, 2012; Namiki et al., 1991; Payne and Hales, 2004; White, 2003). The biosynthesis of these hormones is referred to as steroidogenesis, which is a complex multiple enzyme process utilizing cholesterol as the starting material (Eacker et al., 2008; Fedulova et al., 2010; Jo et al., 2005; Kanzaki and Morris, 1999; King and LaVoie, 2012; Luu-The, 2013; Payne and Hales, 2004; Parker and Schimmer, 1997; Svechnikov et al., 2010). This process is generally carried out in the adrenal glands, placenta and gonads. The development of a thorough understanding of the reactions which occur in steroidogenesis is crucial to the advancement of treatments for a number of medical conditions (Agular et al.,1992; Buren et al., 2002; Diemer et al., 2003; Evain et al., 1976; Fahrenholtz et al, 2013; Hardy et al., 2005; Herrera-Luna et al., 2012, 2013; Jo et al., 2005; Kanzaki and Morris, 1999; Larsson et al., 2011; Reddy et al., 2009; Svechnikov et

al., 2010). A large gap in our current understanding of this process exists in the effects of glucocorticoids on the production of androgens and estrogens. One way to fill this gap is by understanding steroidogenesis as a whole, and then by evaluating the effects of changes in the pathways under specific conditions. This can be accomplished through extensive research and comparison of the mechanisms of steroidogenesis in numerous species.

The existence of alternative metabolic routes for steroidogenesis, and that the preference for a specific pathway can vary between species has long been known (Conley and Bird, 1997; Preslock and Steinberger, 1978). With exponential gains in technology in recent years the ability to thoroughly evaluate this topic is within researchers' grasp. Determination of species-specific pathways can not only provide insight into the physiology of the species, but also provide vital information about pathological conditions like polycystic ovarian disease, castration resistant prostate cancer, hirsutism, and congenital adrenal hyperplasia to name a few diseases characterized by increased androgen production (Conley and Bird, 1997; Lei et al., 2001; Parker and Schimmer, 1997; Svechnikove et al., 2010; White, 2003; Ye et al., 2011). These insights may also elucidate the mechanisms behind infertility and aging. The ability to make comparisons between species and their preferred pathways can allow researchers to choose more appropriate species when planning a project and thus derive more applicable data.

For many years scientists have been compiling data on steroidogenesis. In a study on steroid metabolism in testis tissue, Ruokonen and Vihko (1974) demonstrated similarities between the boar and the human. Researchers studied the conversion of cholesterol to pregnenolone in bovine adrenocortical mitochondrial acetone-dried powder preparations, recognizing the importance of pregnenolone as a cornerstone of androgen and estrogen synthesis in cattle (Burstein and Gut, 1976). A few years later, Preslock and Steinberger (1978) noted a preference for the $\triangle 4$ pathway of testosterone biosynthesis in marmosets. Studies into steroidogenesis have also noted its importance in follicle development in female mammals (Conley et al., 1994), as well as an interrelationship between the function of the gonads and the adrenal glands (Conley and Bird, 1997; deKretser et al., 1998; Eacker et al., 2008; Gummow et al., 2006; King and LaVoie, 2012; Lei et al., 2001; Martel et al., 1994; Parker and Schimmer, 1997; Payne and Youngblood, 1995). More recently researchers have made connections between lowered steroidogenesis and proinflammatory factors, obesity, heart disease, aging, and impaired skeletal health (Blouin et al., 2006; Bonavera et al., 1998; Conley and Bird, 1997; Hong et al., 2004; Karsenty, 2012; Lei et al., 2001; Payne and Youngblood, 1995; Sartorius et al., 2012; Sipahutar et al., 2003; Svechnikov et al., 2010; Wang and Stocco, 2005; White, 2003). These studies indicate that adequate steroid levels are required for normal development and function of the body, whether it is human, horse, or any other species.

Testosterone is a highly studied and well-known androgen. It is required for masculinization and reproductive functions in male mammals, including sperm production (Diemer et al., 2003; Gunasekar et al., 1988; Hess and Franca, 2008; Laughlin et al., 2010; Lopez-Calderon et al., 1991; Payne and Youngblood, 1995; Svechnikov et al., 2010; Yoon and Roser, 2010). Aberrant production levels of testosterone are strongly linked to conditions such as infertility, hyperandrogenism and hypogonadism among others (deKretser et al., 1998; Hardy et al., 2005; Hess and Franca, 2008; Laughlin et al., 2010; Svechnikov et al., 2010; Yoon and Roser, 2010). Recognition of this association has increased research in avian species and their respective methods of steroidogenesis and hormone regulation (London, 2006; Matsunaga et al., 2002; Rangel et al., 2007; Tsutsui et al., 2010; Yu et al., 1995). The elucidation of an array of steroid hormone dependent physiological processes has provided a link between avian and mammal reproductive physiology and a need for further research.

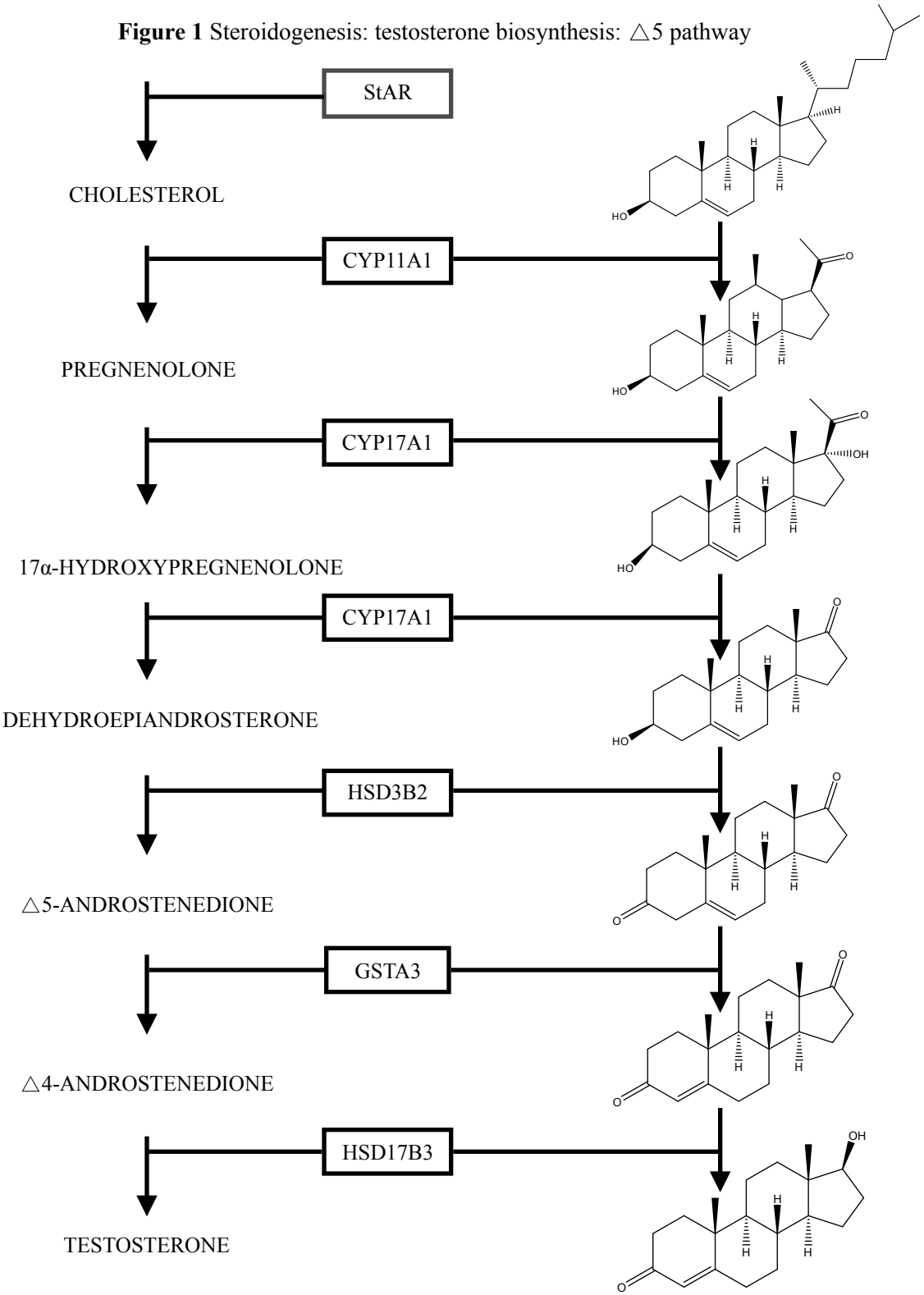
Through elucidation of the mechanisms which regulate testosterone biosynthesis, a better understanding of many complex steroid hormone dependent disorders can be found (Cooke et al., 1991; Gunasekar et al., 1988; Ing, 2005; Johansson and Mannervik, 2001, 2002; Laughlin et al., 2010; Lei et al., 2001; Lopez-Calderon et al., 1991; Luu-The, 2013; Namiki et al., 1991). Many studies have targeted this topic, finding that production of testosterone is largely decreased by the presence of glucocorticoids

(Agular et al., 1992; Badrinarayanan et al., 2006; Cartmill et al., 2006; Hardy et al., 2005; Hu et al., 2008; Ing et al., 2014,2015; Yoon and Roser, 2010). Also products of steroidogenesis, glucocorticoids are produced by the adrenal glands during times of stress (Badrinarayanan et al., 2006; Buren et al., 2002; Gummow et al., 2006; Herrera-Luna et al., 2013; Quax et al., 2013). Glucocorticoids are also widely used as pharmaceuticals to treat conditions involving inflammation (Cooke et al., 1991; Cornelisse et al., 2004; Grady et al., 2010; Liu et al., 2011; Quax et al., 2013). The mechanism by which glucocorticoids impair testosterone biosynthesis is still unknown, but the effects of stress on testosterone production are well documented (Eacker et al., 2008; Hardy et al., 2005; Lopez-Calderon et al., 1991). Many researchers have investigated this glucocorticoid mediated suppression of steroidogenesis in the rat and identified targets for this action (Agular et al., 1992; Cooke et al., 1991; Welsh et al., 1982; Xiao et al., 2010). A few of the targets identified include 3beta-hydroxysteroid dehydrogenase (HSD3B2), steroidogenic acute regulatory protein (StAR), and 17a-hydroxylase (CYP17A1) (Agular et al., 1992; Welsh et al., 1982; Xiao et al., 2010). Continued research into suppression of steroidogenesis via glucocorticoid driven mechanisms will increase scientists' understanding of their actions in health and disease.

Glutathione S-transferase alpha 3

Until recently, the isomerization of Δ5-androstenedione to Δ4-androstenedione, the immediate precursor to testosterone in the biosynthetic pathway (Figure 1), was attributed to the HSD3B2 enzyme (Badrinarayanan et al., 2006; Matsumura et al., 2013; Raffalli-Mathieu et al., 2008). It has now been demonstrated that glutathione S-transferase alpha 3 (GSTA3) is 260 times more efficient at this action than HSD3B2 in humans (Johansson and Mannervik, 2001). GSTA3 is a member of a family of GSTAs which are well known for their actions in detoxification (Benbrahim-Tallaa et al., 2002; Board, 1998; Calvaresi et al., 2012; Gu et al., 2004; Johansson and Mannervik, 2001, 2002; Liu et al., 1993; Matsumura et al., 2013; Morel et al., 2002; Norrgard et al., 2006; Petterson et al., 2002; Prabhu et al., 2004). The GSTAs are isoenzymes of the GST family. Specifically GSTAs are in the cytosolic class, which also includes glutathione S-transferase pi (GSTP) and mu (GSTM) (Dourado et al., 2010, 2014; Fedulova et al., 2010; Higgins and Hayes, 2011; Liu et al., 1993; Norggard et al., 2006; Prabhu et al., 2004). All GSTs act in detoxifying a number of toxic substances by catalyzing their conjugation to glutathione and are found in a number of tissues throughout the body in varying concentrations, with high levels of GSTA and GSTP in the testis (Benbrahim-Tallaa et al., 2002; Fedulova et al., 2010; Hayes et al., 2005; Norggard et al., 2006).

Figure 1 Steroidogenesis: testosterone biosynthesis: △5 pathway



Detoxification by GSTs targets compounds containing an electrophilic carbon, nitrogen, or sulphur atom (Hayes et al, 2005; Sarkar et al., 2001). These toxins include chemical carcinogens, antibiotics, anticancer agents, environmental pollutants, and products of oxidative processes which cause damage to DNA and proteins (Benbrahim-Tallaa et al., 2002; Prabhu et al., 2004; Sarkar et al., 2001; Tars et al., 2010; Ye et al., 2011). GST structure and biochemical composition determine their affinity for a specific substrate and are distinct between the isoenzymes. Their activity has also been shown to be regulated at a transcriptional level, with effects from follicle stimulating hormone (FSH), testosterone, and estradiol, as well as pharmaceuticals like phenobarbital (Benbrahim-Tallaa et al., 2002; Hayes et al, 2005; Higgins and Hayes, 2011). Specifically, treatment of cultures of porcine Sertoli cells with testosterone increased GSTA mRNAs in a dose-dependent and time dependent manner, while treatment with FSH also increased GSTA mRNAs in a dose-dependent manner (Benbrahim-Talla et al., 2002). Both treatments increased expression of GSTA proteins (Benbrahim-Talla et al., 2002). It was also determined that FSH increased the stability of GSTA mRNAs, but testosterone did not (Benbrahim-Talla et al., 2002). With high levels of GSTA gene expression in testis, the known effects of oxidative stress on spermatogenesis, and mounting evidence of the higher efficiency of the GSTA3 enzyme relative to the HSD3B2 enzyme in the isomerization of Δ5-androstenedione to Δ4-androstenedione in testosterone biosynthesis of humans, it is necessary to further understand GSTA gene regulation and actions of the gene products (Calvaresi et al., 2012; Johansson and

Mannervik, 2001, 2002; Larsson et al., 2011; Raffalli-Mathieu et al., 2008; Sarkar et al., 2001; Tars et al., 2010).

The family of GSTA genes is clustered on the same chromosome and their protein products are almost indistinguishable (Johansson and Mannervik, 2001, 2002; Larsson et al., 2011; Morel et al., 2002; Petterson et al., 2002). Further research has revealed that the coding sequence of human GSTA3 mRNA is 92% identical to that of GSTA1 and is 91% identical to that of GSTA2 (Larsson et al., 2011). GSTA4 mRNA shares the least similarity with only 59% identity to the coding sequence of GSTA3 mRNA (Johansson and Mannervik, 2002; Larsson et al., 2011; Morel et al., 2002).

The critical amino acid residues for the 3-ketosteroid isomerase activity of GSTA3, within its 222 amino acid length, were experimentally determined and are presented below in Table 1 for *Homo sapiens* (Dourado, et al., 2014; Johansson and Mannervik, 2002; Petterson et al., 2002). Critical amino acids for other species were determined through comparison of identity by the BLASTX algorithm with the reference sequences from the NCBI database to the human (NM_000847.4) reference GSTA3 mRNA sequence. NCBI sequences (*Equus caballus* NM_001283076.1, *Canis lupus familiaris* XM_532173.4, Capra hircus XM_005696424.1, *Monodelphis domestica* XM_001370694.3, and *Gallus gallus* NM_204818.2) yielding high identity at the protein level were aligned for the location of the human critical amino acids. These residues are located in the active site of the GSTA3 enzyme which has two regions, the

glutathione binding site (G-site) which binds glutathione, and the hydrophobic binding site (H-site) which binds to the steroid substrate (Calvaresi et al., 2012; Dourado et al., 2014; Gu et al., 2004; Johansson and Mannervik, 2001; Petterson et al., 2002; Tars et al., 2010). The amino acid sequences of the GSTAs also share high sequence identity. GSTA1 and GSTA3 differ at 20 of 222 amino acid residues, revealing 91% identity between the two sequences (Johansson and Mannervik, 2001; Larsson et al., 2011). GSTA2 and GSTA4 share 88% identity (26 residues different out of 222) and 54% identity with GSTA3, respectively (Gu et al., 2004; Johansson and Mannervik, 2001; Larsson et al., 2011).

Table 1

Critical amino acids (AA) for 3-ketosteroid isomerase activity of GSTA3. The critical amino acids for the human GSTA3 protein were experimentally determined by Dourado et al., 2014, Johansson and Mannervik, 2002, and Petterson et al., 2002. Other species' critical amino acids were located by the alignment of the human GSTA3 protein translation from NCBI reference sequence NM_000847.4 with protein translations of the reference sequences for horse (NM_001283076.1), dog (XM_532173.4), goat (XM_005696424.1), opossum (XM_001370694.3), and chicken (NM_204818.2).

AA Position:	#9	#10	#12	#111	#208	#216
Homo sapiens	Y	F	G	L	A	A
Equus caballus	Y	F	G	I	G	S
Canis lupus familiaris	Y	F	G	L	L	A
Capra hircus	Y	F	G	L	T	A
Monodelphis domestica	Y	F	G	L	M	A
Gallus gallus	Y	V	G	F	P	V

Despite their high identity, the human GSTA3 enzyme exhibits steroid isomerase activity 10-fold higher than the human GSTA1 enzyme (Calvaresi et al., 2012; Dourado et al., 2014). However, the human GSTA1 enzyme does exhibit some steroid isomerase activity in conjugation with the human GSTA3 enzyme (Gu et al., 2004; Matsumura et al., 2013). Research has demonstrated the human GSTA3 enzyme is activated by steroidogenic factor one (SF-1) which induces a chromosomal conformational change, bringing the promoters of human GSTA3 and GSTA1 into close proximity, at which point the human GSTA3 promoter functions as an enhancer for human GSTA1 (Matsumura et al., 2013). Many studies have investigated the role of the GSTAs in species besides the human. Raffalli-Mathieu and colleagues (2007) cloned and expressed bovine GSTA1 for comparison to human GSTA3. Their investigation demonstrated high amino acid sequence identity and selective expression in steroidogenic organs, four out of five residues critical for steroid isomerase activity were conserved, but the enzyme's catalytic efficiency in steroid isomerization was 100 times lower than that of human GSTA3. However, the catalytic efficiency in steroid isomerization of bovine GSTA1 was equal to that of bovine HSD3B.

The catalytic efficiency of the porcine GSTA2 enzyme as a steroid isomerase was evaluated in another study (Fedulova et al., 2010). In this investigation the porcine GSTA2 enzyme, like the bovine GSTA1 enzyme, was cloned, expressed, and its catalytic activity was compared to the human GSTA3 enzyme. Porcine GSTA2, unlike bovine

GSTA1, was determined to be an analog of the human GSTA3 enzyme (Fedulova et al., 2010). There were significant similarities between human GSTA3 and porcine GSTA2 in steroid isomerase activity and selective expression pattern for steroidogenic tissues. The isomerase activity of porcine GSTA2 was lower than that of human GSTA3, but higher than human GSTA1 steroid isomerase activity. It was demonstrated that porcine GSTA2 and human GSTA3 have 84% identity in their amino acid coding sequences and both demonstrate inhibition by the endocrine disruptor tributyltin chloride (Fedulova et al., 2010). Characterization of the GSTA3 gene products is far from complete and many other species may demonstrate conservation of the GSTA3 gene and its isomerase activity.

The activity and specificity for these residues was investigated in human GSTAs by cloning and bacterial expression procedures. Specific point mutations switched GSTA2 residues into GSTA3 protein at the specified critical positions (Johansson and Mannervik, 2002; Petterson et al., 2002). Activity of the GSTA3 enzyme with the steroid substrate was determined to be most affected by mutation of residues at amino acid position 10 phenylalanine (F), 111 leucine (L), and 216 alanine (A). Substitution of residue 216 alanine with serine (S) showed little effect on isomerase activity, but mutations in residues 10, 111, and 216 combined dropped the enzymatic activity to 0.8% that of the wild-type (Johansson and Mannervik, 2002). A single nucleotide base change in a codon for the amino acid serine can elicit the change of that serine to a threonine

(T), proline (P), or alanine (A), thus creating an isozyme if the enzyme function is unaltered. Amino acid residues with similar chemical properties to those of the native human GSTA3 protein are unlikely to alter overall function of the enzyme.

Although the sequences of the 12 human GSTA genes and pseudogenes are well documented, the GSTA gene sequences of other species are usually omitted during genome sequence assembly (Matsumura et al., 2013; Morel et al., 2002). This tendency is due to the sequence similarity of the GSTAs and the use of computer programs to assemble the genome from short sections based on paired-end reads and overlapping regions. Often, the whole genome shotgun sequencing approach is used to generate millions of sequence reads with a high level of redundancy (6X or more) in order to improve accuracy of assembly. Short, repeated or highly similar sequences which cannot be organized into a contig are considered inconsistencies or gaps and are left out of the sequence for further review. Elucidation and documentation of these sequences will allow for further analysis of the GSTA3 mechanism of action in testosterone biosynthesis, and provide sequence identity data between species. Across species comparison is an extremely valuable commodity when determining appropriate experimental models. Previous research has indicated that the glucocorticoid dexamethasone represses expression of the GSTA3 gene in horse testis (Ing et al., 2014, 2015). Studies involving exogenous glucocorticoids like dexamethasone have also clearly indicated down-regulation of testosterone biosynthesis, adding evidence to the

hypothesis that these effects are mediated by the repression of the GSTA3 gene (Barth and Bowman, 1994; Cartmill et al., 2006; Ing et al., 2015).

Glucocorticoids

Glucocorticoids are cholesterol derivatives produced by steroidogenesis in the adrenal glands. They act as endocrine regulators and are well known for their actions during stress (Cooke et al., 1991; Hardy et al., 2005; Kizaki et al., 1998; Liu et al., 2011; Lopez-Calderon et al., 1991; Schmidt et al., 2010; Surjit et al., 2011). Functions of this family of steroids includes regulation of metabolism, embryogenesis, inflammatory responses, programmed cell death, cell proliferation and differentiation, behavior, neurobiology, and homeostasis (Bladh et al., 2005; Gold et al., 2012; Kizaki et al., 1998; Liu et al., 2011; Schmidt et al., 2010; Smith et al., 2010; Smoak et al., 2006). Glucocorticoids act on the glucocorticoid receptor (GR) and are regulated at the tissue level by two enzymes, HSD11B1 and 2 (Herrera-Luna et al., 2012, 2013; Hu et al., 2008; Xiao et al., 2010). In humans the HSD11B2 enzyme acts in the conversion of cortisol, the active glucocorticoid, to cortisone, a less active glucocorticoid, while HSD11B1 catalyzes the reverse reaction (Bladh et al., 2005; Liu et al., 2011; Yick-Lun So et al., 2007). The glucocorticoid receptor gene is expressed in most cells and is very highly expressed in the Leydig cells of the testis (Herrera-Luna et al., 2012; Hu et al., 2008; Ing, 2005; Liu et al., 2011; Lopez-Calderon et al., 1991; Quax et al., 2013).

A number of synthetic glucocorticoids have been developed as pharmaceuticals in order to take advantage of their many effects on physiology and pathology. Synthetic glucocorticoids include prednisone, prednisolone, and dexamethasone. These drugs treat a wide variety of conditions involving inflammation. They exert their effects by binding the glucocorticoid receptor, which mediates transactivation and transrepression of target genes by binding regulatory regions as well as interacting with other transcription factors (Liu et al., 2011; Smoak et al., 2006; Surjit et al., 2011; Yick-Lun So et al., 2007). Specifically, dexamethasone can both up and down regulate genes involved in regulating inflammation, cell growth, and cell death (Liu et al., 2011). It can also exert post-transcriptional gene regulation inducing expression of microRNAs and decreasing the stability of specific mRNAs (Ing, 2005; Liu et al., 2011; Smith et al., 2010).

Dexamethasone treatment of stallions causes an acute decrease in serum testosterone concentrations (Ing et al., 2014, 2015). Weeks later, there is a decrease in the number of normal, motile sperm (Danek, 2004). In other species, dexamethasone induced abnormalities include sperm morphology and biochemical composition (Barth and Bowman, 1994; Danek, 2004; Ing et al., 2014, 2015). These effects are transient. The risk of decreased testosterone and semen abnormalities resulting in decreased fertility due to dexamethasone treatment elicits much interest from those in the animal breeding industries, causing a push for further elucidation of the mechanism behind these effects.

The Leydig cells are the primary site of testosterone synthesis and, thus, provide a link between the inhibitory effects of glucocorticoids on testosterone production because of the high concentration of GRs located there. Also, the anti-inflammatory effects of glucocorticoids are induced by the up-regulation of inhibitors of inflammatory mediators and the repression of cytokine gene expression by transcriptional regulation and mRNA destabilization (Bladh et al., 2005; Grady et al., 2010; Ing, 2005; Quax et al., 2013; Smith et al., 2010). Dexamethasone, a frequently prescribed glucocorticoid, has been demonstrated to be 30 times as potent as endogenous cortisol, as well as having a longer duration of action than that of prednisolone, another frequently prescribed glucocorticoid (Cornelisse et al., 2004; Grady et al., 2010; Liu et al., 2011). Dexamethasone is generally prescribed for inflammatory diseases including encephalitis, arthritis, and recurrent airway obstruction in horses. In humans, glucocorticoids are prescribed for treating asthma, rheumatoid arthritis, brain tumors, prostate cancer, and some side effects of chemotherapy in humans, as well as many other conditions (Cornelisse et al., 2004; Grady et al., 2010; Smoak et al., 2006).

Dexamethasone is commonly used in research studies which focus on the physiology of the stress response of a research organism (Agular et al., 1992; Buren et al., 2002; Cartmill et al., 2006; Haffner et al., 2009; Herrera-Luna et al., 2012, 2013; Ing et al., 2014, 2015; Smoak et al., 2006). Researchers have demonstrated that dexamethasone treatment of stallions reduces the concentration of testosterone in the

circulation by 60% within 12 hours post-injection (Ing et al., 2014, 2015). It was also demonstrated that GSTA3 mRNA concentrations in testes from that study decreased two fold at 12 hours post-injection (Ing et al., 2014). Results with stallions clearly indicate that dexamethasone administration decreases testosterone and later, reproductive efficiency (Danek, 2004). A study conducted in bulls by Barth and Bowman (1994) to analyze the effect of testicular heating and stress on spermatogenesis utilized dexamethasone as their method of mimicking stress. This study showed that bulls treated with dexamethasone displayed significantly ($p < 0.05$) lower testosterone concentrations. A marked increase in sperm defects was also displayed in dexamethasone treated bulls (Barth and Bowman, 1994). This breadth of information provides ample justification for further research into steroidogenesis, the testosterone biosynthesis pathway, and the expression and regulation of the GSTA3 gene.

Species Divergence and Gene Conservation

In-depth analyses of the compiled genome sequences for multiple species through the use of public databases like NCBI allows for the elucidation of orthologous genes. Utilization of molecular biology tools can organize data from genomic and genetic analysis projects. The increasing number of published genomes functions as a useful resource for compiling the genomes of previously understudied species (Dong et al., 2013, Frings et al., 2012; Hillier et al., 2004; Sawai et al., 2010, Skinner et al., 2009;

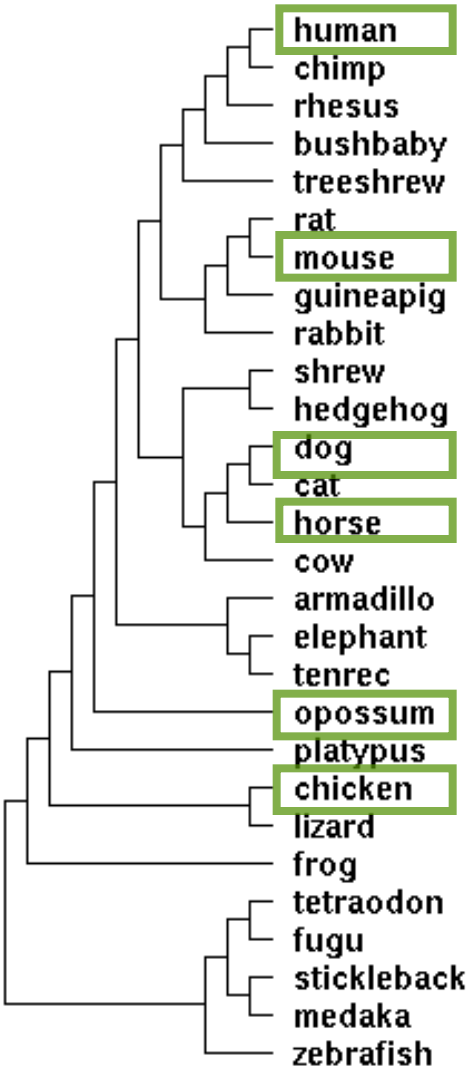
Skoglund et al., 2011; Wade et al., 2009). Numerous studies utilizing comparative genomics to determine the location, function and interactions of genes have been recently carried out. Das and colleagues (2012) analyzed genomic imprinting in *Monodelphis domestica* as compared to the human and mouse in several specific genes and found a large degree of consistency. A recent study focusing on the evolution of the marsupium utilized complete mitochondrial genome for phylogenetic analysis of morphological features typical of marsupials to verify the classification of *Monodelphis domestica* and *Myrmecopius fasciatus* (Hermick et al., 2013). Orlando and colleagues (2013) exploited the vast availability of equid fossil records for a comparison between the Middle Pleistocene, Late Pleistocene and multiple modern domestic horse breeds in order to further our knowledge of the evolution of *Equus caballus*. Using this analysis it was proposed that the most recent common ancestor to all modern *Equus caballus* lived 4.0 to 4.5 million years ago. Connections were demonstrated between horse population evolution and environmental adjustments of grassland availability and temperature. These studies demonstrate the vast array of knowledge which can be achieved using cross species genetic and genomic comparisons, including analyzing the occurrence of gene conservation.

Comparative genomics studies typically utilize closely related species and divergent species are avoided because the data obtained would be more difficult to assess accurately for similarity. However, this does not mean that divergent species will not have a specific gene of interest. It is generally accepted that chickens (*Gallus*

gallus), and mammals diverged an estimated 310 million years ago (Shaul and Graur 2002). This estimate, like all others, varies based on the specific study and methods used. For this reason, Shaul and Graur (2002) did an analysis of these inconsistencies based on the calibration points used, which resulted in the identification of a new estimate of 393 million years ago with a 95% confidence interval of 315- 471 million years. Placental and non-placental mammals are estimated to have diverged 150 million years ago (Das et al., 2012). This demonstrates that opossums are more closely related to humans than chickens. The rodent - primate divergence date has been estimated at 110 million years ago which fully supports the acknowledged relationship proximities between birds and humans or mice and humans (Shaul and Graur, 2002). It is unclear whether rodents express a GSTA gene product with 3-ketosteroid isomerase activity. A phylogenetic tree of relationships of vertebrate species with assembled genomes is displayed in Figure 2.

Examples of comparative genomics are not all necessarily recent, but the current availability of next generation sequencing and physical mapping options greatly increases the ease of execution and accuracy of this type of project. The conservation of microsatellite sequences between cattle (*Bos taurus)*, goats (*Capra hircus)* and fallow deer (*Cervus dama)* was demonstrated by Laurent Pepin and colleagues (1995). This study revealed that roughly 40 percent of the microsatellites developed for and utilized in cattle genomics can be employed in the investigation of the *Capra hircus* genome and provide useful information in regards to the genetic diversity of *Artiodactyla*.

Figure 2 Phylogenetic relationships of vertebrate species with assembled genomes. Species investigated here are boxed in green. The goat is not shown. Image taken from UCSC Genome Wiki, 2014 which was adapted from Miller et al., 2007. Full information on citation in references.



Recently a study of the caprine genome by Dong and colleagues (2013) has provided a more thorough understanding of the genetic basis of complex traits and the effects of breed on phenotype. Today there are more than 830 million goats of more than 1,000 breeds kept around the world for purposes including milk, meat, and fiber production. However, according to Dong and colleagues (2013) research has previously been hindered by the lack of a complete reference genome sequence despite the goat's agricultural and biological importance. Many other studies point out the importance of decoding the genomes of different species for the purpose of understanding gene function and expression, especially of those complex pathways involved in development and disease (Dong et al, 2013; Frings et al., 2012; Hermick et al., 2013; Hillier et al., 2004; Sawai et al., 2010; Skinner et al., 2009; Wade et al., 2009).

Elucidation and comparison of genomes between different breeds of domestic dogs have also been identified as important areas of research for their ability to impart an understanding of the relationships between phenotypic diversity and morphological, physiological and behavioral traits, better known as breed characteristics (Lindblad-Toh et el., 2005; Skoglund et al., 2011). Lindblad-Toh and colleagues (2005) utilized comparative genomics for insights into this topic and were able to map and verify numerous SNPs which will provide for more accurate location of genes responsible for diseases and traits of interest. This study also made comparisons between the dog, human and mouse genomes, elucidating significant regions of similarity and providing evidence for the ability of cross species comparisons to identify genes involved in

physiological processes and causes of disease. These studies are just a few of the numerous examples which identify the suitability of cross species gene comparisons. Furthermore, demonstration of conservation between multiple species, especially those of a divergent nature, allows for identification of unique genes and functional non-coding sequences, as well as increased accuracy of evolutionary relationships. For these reasons the project described here utilizes a range of species of varying divergence in its investigation of the GSTA3 gene. Demonstration of the conservation of this gene and its function over numerous species solidifies our understanding of its role in physiology and allows for selection of a specific species for laboratory experiments.

CHAPTER III

MATERIALS AND METHODS

Tissue Samples

Tissue samples for this study were donated to the project by the Texas A&M College of Veterinary Medicine from animals which were sacrificed for teaching purposes. Tissues consist of testes from multiple species including horse (*Equus caballus),* dog *(Canis lupus familiaris),* gray short-tailed opossum *(Monodelphis domestica),* chicken *(Gallus gallus)* and goat (*Capra hircus)*. Multiple tissue samples from *Equus caballus* and *Canis lupus familiaris* consisting of adrenal gland, cerebrum, heart, hypothalamus, kidney cortex, liver, lung, mammary, ovary, skeletal muscle, small intestine, spleen, urinary bladder, and uterus were also used. All dog tissues except the ovary and testis came from a twelve year old female black and tan hound. The other tissues were collected from a seven year old and a ten year old red bone hound, while the testis tissue was collected from a two year old bulldog. Equine tissues were collected from a sixteen year old paint mare and a four year old quarter horse cross stallion.

RNA Extraction

In order to isolate the desired mRNA sequence from the tissue, the RNA must first be extracted. Tissue samples were snap frozen and stored at -80ºC. 0.5 g of each tissue sample was homogenized in 5 ml of room temperature Tripure solution (Roche Applied Science, USA) with a tissue homogenizer (Kinematica GmbH distributed by Brinkman Instruments, Westbury NY). After the tissues were homogenized, the homogenized tissue samples were incubated at room temperature for five minutes, chloroform was added and samples were shaken vigorously. Then the samples were incubated again at room temperature for five minutes. A centrifuge was used to separate the phases and the aqueous phase was removed via pipette to a clean sample tube. RNA was precipitated from the aqueous phase by the addition of isopropanol and an incubation period. Samples were again centrifuged. This time a pellet formed at the bottom of the sample tube. Washing the pellets in 75% ethanol removed remaining salts. The pellets were briefly air dried. Finally, the RNA pellets from the tissue samples were dissolved in diethylpyrocarbonate (DEPC) treated H_2O and then quantified for RNA concentration and purity using a Nanodrop Spectrophotometer, as well as assessed for quality on a 2100 Bioanalyzer (Agilent; Santa Clara, CA).

Concentration of RNA in each sample as determined by the Nanodrop spectrophotometer was used to calculate the volume of each sample to be used in the reverse transcription reaction. Data obtained through the use of this process also yields a

measure of purity. The spectrophotometer measures the absorbance of the sample at 260 nm to determine the concentration of nucleic acids in solution, this is known as the A_{260}. For RNA an A_{260} of 1.0 is equal to a concentration of 40 μg ml^{-1}. The spectrophotometer can also act as an indicator of purity by measuring the A_{280}, thus providing quantification by the A_{260}/A_{280} ratio. Pure RNA would have a value near 2.0 for this ratio. The 2100 Bioanalyzer by Agilent uses capillary electrophoresis to measure RNA integrity. This is done by assessing intact RNAs as well as the presence or absence of degradation products. The measure of integrity is quantified in the RNA integrity number (RIN). The optimal RIN with no degradation products present would have a value of 10, whereas a sample which is almost completely degraded would have a value of 1. Samples from this project were run on the 2100 Bioanalyzer Nano chip and had RIN values ranging from 7.7 to 9.4.

Reverse Transcription

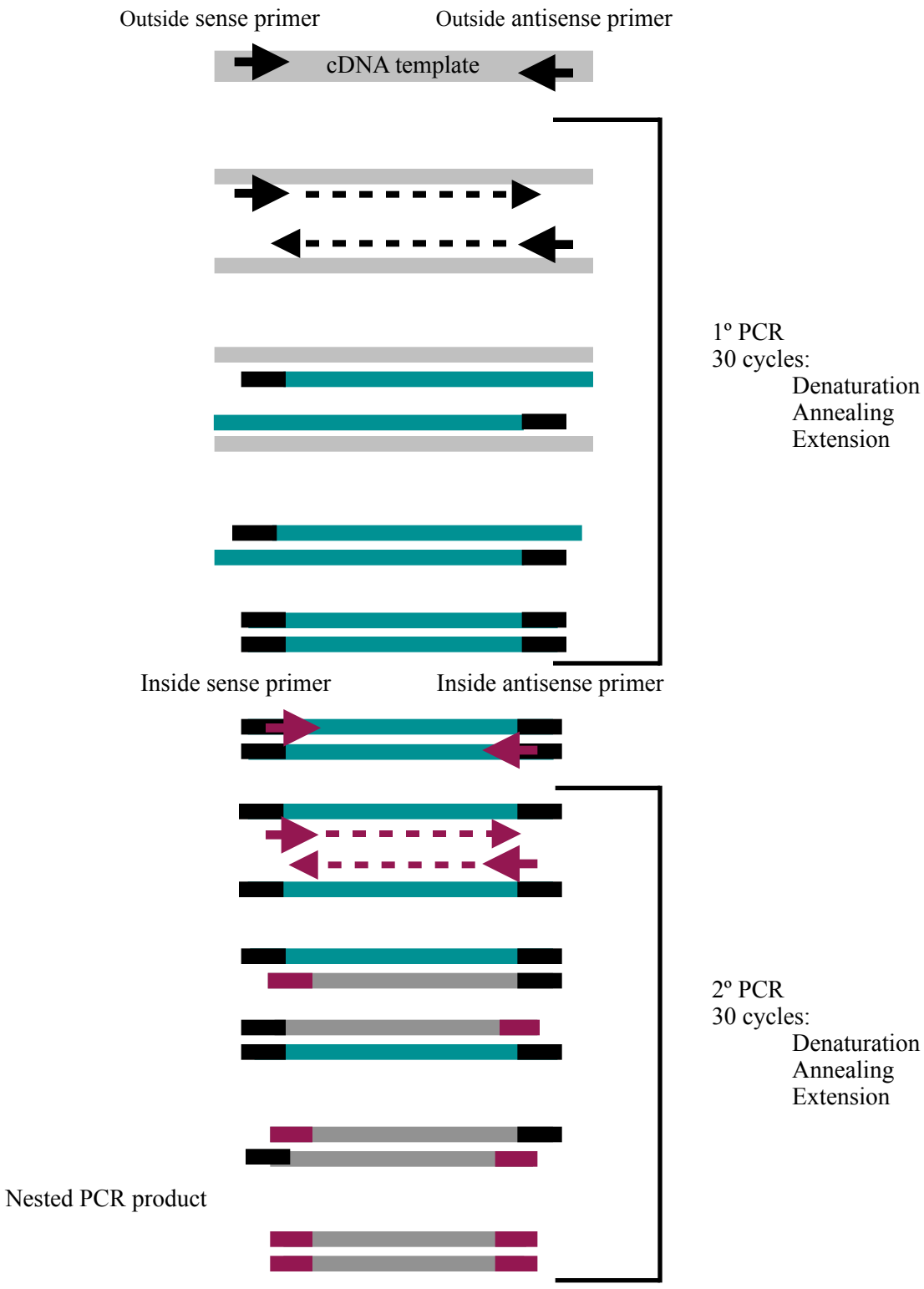
Using the isolated RNA, complementary DNA (cDNA) was produced by reverse transcription. The cDNA is necessary for amplification by polymerase chain reaction (PCR). Using the data from the Nanodrop the volume to be used of each tissue RNA sample was calculated. RNA sample amounts for cloning used 500 ng of RNA and samples for real time quantitative PCR (qRT-PCR) used 100 ng of RNA. Two master mixes were created, one master mix consisted of a random octamer and dT_{20} primers, and a second master mix consisted of 5X first strand buffer, 0.1M DTT, dNTP mix, and

Superasin (Life Technologies, Thermo Fisher Scientific, Inc.). Master mix one, nuclease-free H_2O (Life Technologies, Thermo Fisher Scientific, Inc.) and the RNA from each tissue sample was combined in a micro-centrifuge tube, vortexed, centrifuged for a short time, incubated in a heating block at 70ºC for five minutes, quickly chilled on ice and then centrifuged again. Master mix two was then added to each sample tube, mixed gently and incubated at room temperature for two minutes. The Superscript II Reverse Transcriptase (Life Technologies, Thermo Fisher Scientific, Inc.) was then added, mixed via pipette and incubated at room temperature for 10 minutes. The samples were then incubated at 42ºC for three hours. Afterwards, the Superscript II Reverse Transcriptase was inactivated by heating at 70ºC for 15 minutes.

Nested PCR

Polymerase Chain Reaction (PCR) is utilized for amplification of cDNA. In this process the cDNA is denatured so that a primer can bind a specific target sequence and then prime polymerization by Taq DNA polymerase. This process is repeated for 30 to 40 cycles to generate a suitable amount of cDNA product. The term "nested" is utilized when referring to multiple sets of PCR with different primers for each set. The final primers must target a section of the intended product which is "inside" of the previous set, or the "outside" primers (Figure 3). This technique is utilized in an attempt to increase the sensitivity and fidelity of the PCR reaction.

Figure 3 Diagram of nested polymerase chain reaction



Nested PCR was chosen as the preferred protocol because of its tendency to increase the sensitivity of PCR and reduce the generation of non-specific products. For this protocol, two sets of PCR primers were created: one set of forward and reverse "outside" primers and one set of forward and reverse "inside" primers. Creation of these primers was accomplished with analysis of the human GSTA3 mRNA (NM_000847.1) sequence which is well documented in the NCBI database, as well as by comparison to previous documentation of the equine GSTA3 mRNA sequence also located in the NCBI database (KC512384.1). Separate sets of primers were made for cloning into each vector. The pET-21a vector utilizes the BamHI endonuclease sequence (GGATCC) for the initiation of translation for expression and the insert sequence must be in frame for this to occur. In order to ensure that our cDNA inserts would ligate in frame with the BamHI site we included the EcoRI endonuclease sequence (GAATTC), which is located directly next to the BamHI endonuclease site, at the 5' end of the sense primers. The Xho I endonuclease sequence (CTCGAG) was included at the 5' end of the inside antisense primer to increase success of the ligation reaction. Primers were ordered from Integrated DNA Technologies (IDT) and their sequences are in Table 2 for cloning into pCR2.1 and Table 3 for cloning into pET-21a.

The primary PCR reaction was performed with the set of "outside" primers designated for each species, the previously made cDNA template from each species, and a master mix consisting of nuclease free H_2O, 10X Taq buffer, Ex Taq enzyme (Takara Bio Inc., Otsu, Shiga, Japan) and 2.5mM dNTPs. Final sample volume was 50 µL. PCR

Table 2
PCR primer sequences for cloning cDNAs into pCR2.1 (5'- 3') GSTA3

Species	Primer Set	Orientation	Sequence
Canis lupus familiaris	Outside	Sense	GGAGACTGCATCATGGCAGTGAAGCCCATG
		Antisense	AGGAGATTGGCCCTGCATGTGCT
	Inside	Sense	ATGGCAGTGAAGCCCATGCTTCACTACTTCAATGG
		Antisense	GCTGGCATCCATTCCTGTTCAGTTAAT
Capra hircus	Outside	Sense	GGAGACTGCATCATGGCAGTGAAGCCCATG
		Antisense	TCAAATTTGTCCCAAACAGCCCC
	Inside	Sense	ATGGCAGTGAAGCCCATGCTTCACTACTTCAATGG
		Antisense	CCCCGCCAGCCGCCAGCTTTATTAAAACTT
Monodelphis domestica	Outside	Sense	GGAGACTGCATCATGGCAGTGAAGCCCATG
		Antisense	TGTGTTTAAGAAACACAGAGTCA
	Inside	Sense	ATGGCAGTGAAGCCCATGCTTCACTACTTCAATGG
		Antisense	TCATCAAACTTGAATATCTTCTTTGCC

Table 3
PCR primer sequences for cloning cDNAs into pET-21a (5'- 3') GSTA3
Lowercase letters designate an endonuclease sequence.

Species	Primer Set	Orientation	Sequence
Canis lupus familiaris	Outside	Sense	gcgaattcCCAGAGACTACCATGGCGGGGAAGCCCAAG
		Antisense	TCTCAGGAGATTGGCCCTGCATG
	Inside	Sense	gcgaattcATGGCGGGAAGCCCAAGCTTCACTACTTCAATGG
		Antisense	cgctcgagCTGGGCATCCATTCCTGTTCAGTTATCCT
Capra hircus	Outside	Sense	gcgaattcAGAACTGCTATTATGGCAGGGAAGCCCAT
		Antisense	TCAAATTTGTCCCAGACAGCCCC
	Inside	Sense	gcgaattcATGGCAGGGAAGCCCATTCTTCACTATTTCAATGG
		Antisense	cgctcgagCCCCGCCAGCCGCCAGCTTTATTAAAACTT
Monodelphis domestica	Outside	Sense	gcgaattcGAATGGAAGATCATGTCTGGGAAGCCCAT
		Antisense	TTGCATTACTTAGAACTCTTCCTGAATATTCAGCT
	Inside	Sense	gcgaattcATGTCTGGGAAGCCCATACTTCATTACTTCAATGG
		Antisense	cgctcgagTCAGCTCATATGTTTAAGAAACAAGTCTTC
Gallus gallus	Outside	Sense	gcgaattcGGAGCTCAGAAGCATGTCGGGGAAGCCCAGG
		Antisense	TCCCAGCACTTTGGGACTTAGTG
	Inside	Sense	gcgaattcATGTCGGGGAAGCCCAGGCTCACCTATGTCAATGG
		Antisense	cgctcgagGTGAAGTTAACGCTGCACTCAGTTTAG

was performed in the thermal cycler (GeneAmp PCR System 9600, PerkinElmer, Norwalk, CT) for 30 cycles of 94°C denaturation for 15 seconds, 45°C annealing for 30 seconds, and 72°C extension for one minute. Finally, there was a 72°C hold for five minutes. The secondary PCR reaction was performed with the set of "inside" primers for each species, one tenth (5 μL) of the primary PCR reaction products and a similar master mix as the first. Secondary PCR reaction samples were placed in the thermal cycler and run on the same settings as the Primary PCR reaction. Secondary PCR reaction products were combined with 10X DNA dye and analyzed via gel electrophoresis. A 1% agarose gel and a 0.8% low melting temperature (LMT) agarose gel made with ethidium bromide were run in 1X TAE buffer, with Lambda HindIII EcoR1 marker and 10X DNA dye at 100 volts for 45 minutes. Ethidium bromide-staining allowed visualization of the expected cDNA products, which were photographed. Products were expected to be 670-700 base pairs. Bands were cut from lanes in the 0.8% LMT gel displaying expected cDNA products for use in ligation reactions.

Plasmid Vector Cloning with pCR 2.1

Ligation in pCR 2.1

The cDNA PCR product bands cut from the 0.8% LMT agarose gel for each species was melted at 70°C for ten minutes and then cooled to 37°C. They were then

ligated to pCR 2.1 for use in the transformation of One Shot TOP10F' chemically competent E. coli (TA Cloning Kit K2030-40, Life Technologies, Thermo Fisher Scientific Inc.). Each ligation reaction was of a 10 µl volume, and were performed at 15°C. The concentrations of the reagents in each reaction varied depending on whether it was the maximum, minimum, or negative control. Maximum reaction tubes received 3 µl kit H_2O, 1 µl 10X ligation buffer, 2 µl vector DNA, 3 µl cDNA insert, and 1 µl T4 DNA ligase. Minimum reaction tubes received 5 µl kit H2O, 1 µl 10X ligation buffer, 2 µl vector DNA, 1 µl cDNA insert, and 1 µl T4 DNA ligase. The negative control tubes received 6 µl kit H_2O, 1 µl 10X ligation buffer, 2 µl vector DNA, no cDNA insert, and 1 µl T4 DNA ligase. All reaction tubes were then placed in the 15°C block to incubate overnight.

Transformation

After the ligation reactions, samples ligated to pCR 2.1 were utilized in transformation reactions with E. coli and spread on LB-Ampicillin plates and incubated at 37°C overnight for colony growth. Before the plates were spread with the transformation reactions they were spread with 80 µl 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGAL) and 4 µl 20 mg/ml Isopropyl β-D-1-thiogalactopyranoside (IPTG). The plates were then incubated at 37°C while the transformation reactions were prepared. Transformation reactions were prepared identically by placing 1 µl of each ligation into a specified competent cell tube and

incubated on ice for 30 minutes. Competent cell tubes were then heat shocked at 42°C for 20 seconds and placed on ice for two minutes. Finally 950 µl of room temperature SOC broth was added to each competent cell tube and they were placed on a rocker in a 37°C incubator for one hour. The plates were then spread with 200 µl of their designated competent cell tube and incubated at 37°C overnight. The next day colonies were counted and colonies from these plates were randomly chosen for overnight LB broth cultures. Each colony collected from the LB-Ampicillin plates was cultured overnight in 5 ml LB-Ampicillin broth at 37°C with strong agitation.

Plasmid Purification

The plasmid DNA from these cultures was purified using the QIAprep Spin Kit from Qiagen. To accomplish this the overnight cultures were centrifuged and the supernatant was removed and discarded. The bacterial pellets were resuspended with 250 µl of buffer P1 and transferred to 1.5 ml micro centrifuge tubes. 250 µl of buffer P2 was added to each sample tube and the tubes were then inverted four to six times to mix. Next, 350 µl of the buffer N3 was added and the sample tubes were inverted four to six times to mix. Samples were then centrifuged for ten minutes at 13,000 rpm. The supernatant was then pipetted in QIA prep spin columns and centrifuged for 60 seconds. The flow through was discarded and the column was washed with 500 µl of buffer PB. Sample tubes were then centrifuged for 60 seconds and the flow through was again discarded. Next, the column was washed with 750 µl of buffer PE and again centrifuged

for 60 seconds. In order to remove any residual buffer the samples were centrifuged again for 60 seconds, and then the the column was placed into a clean 1.5 ml micro centrifuge tube. Finally, 50 µl of buffer EB was placed into the center of each column and allowed to set for one minuted and then centrifuged for one minute.

The purified plasmid DNA samples were digested with EcoR1. This was accomplished by combining 10 µl of plasmid sample with 7 µl nuclease free H_2O, 2 µl 10X H buffer, and 1µl EcoRI. Reactions were incubated at 37^{o}C for one hour. The restricted plasmid DNA products were analyzed by gel electrophoresis. Samples were dyed with 10X DNA dye. A 1% agarose gel containing ethidium bromide in 1X TAE buffer was loaded with the marker Lambda HindIII EcoR1 and the samples. The gel was then run at 100 volts for 45 minutes, and desired products were visualized under UV light and photographed.

Plasmid Vector Cloning with pET-21a

One set of *Canis lupus familiaris, Capra hircus, Gallus gallus* and *Monodelphis domestica* testis derived cDNA was cloned into a plasmid expression vector, pET-21a (Novagen). Nested PCR was utilized in this process as described previously. Primers used are those shown above in Table 3. The PCR amplified product and the pET-21a vector were restricted with EcoR1 and Xho1 endonucleases. This process was

completed with micro-spin columns pre-spun to remove the buffer. The secondary PCR product was spun through the column and then the column was discarded. Next, 2 µl of 10X NeBuffer 4 (NEB4) and 0.2 µl of 10mg/ml bovine serum albumin (BSA) was added to the tube. EcoR1 and Xho1 endonucleases were added in volumes of 1µl each and the tube was incubated at 37°C for three to four hours. The samples were dyed with 10X DNA dye and then analyzed on both 1.0% agarose and an 0.8% LMT agarose gel in 1X TAE buffer with Lambda Hindi III EcoR1 as the marker. The gels were run at 100 volts for 45 minutes and visualized under UV light. Bands of expected size were visualized in the 1.0% agarose and thus bands were able to be cut for use in a ligation reaction from the 0.8% LMT agarose gel.

The expression vector pET-21a was utilized to complete a ligation reactions for each species. Both the cDNA bands cut from the 0.8% LMT agarose gel and the pre-digested vector were melted at 70ºC for ten minutes before being transferred to a 37ºC hold. Using 8µl of the kit H_2O and 2µl 10X ligation buffer for each sample a master mix was made on ice. Each new sample tube was well labeled and placed at 37ºC as well as a negative control (NC) tube. 8µl of kit H_2O was added to the NC tube and 5µl was added to each new sample tube at 37ºC. Next, 1µl of pET-21a vector was added to each tube. Three micrometers of each cDNA sample was added to its respective new sample tube, and the tubes were mixed gently. Finally, 10µl of the master mix waiting on ice was added to each tube and the samples were then incubate at 15ºC overnight. LB-agar

plates for colony growth were not spread with XGAL and IPTG because pET-21a contains a lacI region instead of a lacZ region as pCR 2.1 does. Vector pET-21a also demonstrates a higher ampicillin sensitivity and thus specific plates were created for each set of samples depending on which vector they were ligated to.

The pET-21a clones were purified in the same manner as the pCR2.1 clones except they were digested with both EcoR1 and Xho1. A master mix of 5.8 µl nuclease free H_2O, 2µl 10X NE Buffer 4 (NEB4), 0.2µl 10mg/ml bovine serum albumin (BSA), and 1µl of each restriction endonuclease for each sample was made. This was then combined with 10 µl of the purified plasmid DNA and incubated at 37ºC for three to four hours. Restricted plasmid DNA products were analyzed by gel electrophoresis on a 1% agarose gel containing ethidium bromide in 1X TAE buffer with the Lambda HindIII EcoR1 marker and desired products were visualized under UV light and photographed.

DNA Sequencing with PCR

Plasmid miniprep samples displaying desired cDNA products in the gel photograph of the restriction digests were sequenced with PCR. This was accomplished with Big-Dye mix (Applied Biosystems), nuclease free H_2O (Life Technologies, Thermo Fisher Scientific, Inc.), M13 forward (5'-TGTAAAACGACGGCCAGT-3') or reverse primer (5'-CAGGAAACAGCTATGAC-3'), and the DNA template from the plasmid

miniprep samples selected. Sequencing of the pET21a clones was completed separately with the T7 promoter primer (5'-TAATACGACTCACTATAG-3') and the T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3'). These were mixed in a thin-walled PCR tube and placed into the PCR machine for 99 cycles of 96^{o}C denaturation for 20 seconds, 50^{o}C annealing for 10 seconds, and 60^{o}C extension for four minutes. PCR products then underwent centrifugation in a mini-spin chromatography tube for the removal of unincorporated nucleotides and were submitted for automated sequencing by the Texas A&M University Gene Technologies Laboratory. Samples were denatured at 95ºC and sequenced with capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer. The capillary length was 50 cm and 3130 POP-7 TM polymer was used for sequencing.

Sequence Analysis

Once sequenced the sample nucleotide sequences were retrieved from the Texas A&M University Gene Technologies Laboratory website. The experimentally obtained GSTA3 mRNA sequences and chromatograms were then labelled with their miniprep number, species, and GSTA3. The EcoR1 site (GAATTC) of the pCR2.1 vector and the "inside" PCR primers were located and marked in the sequence. The NCBI GenBank database records of the human GSTA3 (NM_000847.1) mRNA reference nucleotide sequence was used to compare to the experimentally obtained nucleotide sequences from other species. The equine GSTA3 mRNA sequence (KC512384.1) was accessed with

this source and used as a reference for sequence comparisons as well. Experimentally derived sequences were also evaluated for identity to documented GSTA3 genes using NCBI's basic local alignment search tool (BLAST) to check for significant alignments to other NCBI reference sequences.

The sequence from each species with the highest identity to the NCBI reference sequences NM_000847.4 and KC512384.1 was chosen and translated to its amino acid sequence. The antisense sequences chosen were run through the reverse complement generator at bioinformatics.org before they were translated. Translation to the amino acid sequence was accomplished by the use of the ExPASy translate tool available online. To ensure that the product sequence was in fact GSTA3 and not one of its very similar GSTA family members, the six identified amino acids were located in each sequence (Table 1.). Following this analysis chosen sequences were submitted to NCBI GenBank and can be accessed there. Plasmid maps detailing the restriction sites and specific plasmid vectors, as well as sequence orientation and length were also created for each GSTA3 sequence submitted (Appendix C, Figures C-1 to C-7).

Real Time Quantitative Polymerase Chain Reaction

Real Time Quantitative Polymerase Chain Reaction (qRT-PCR) was used with relative quantification procedures on the RNA samples from multiple tissues in order to

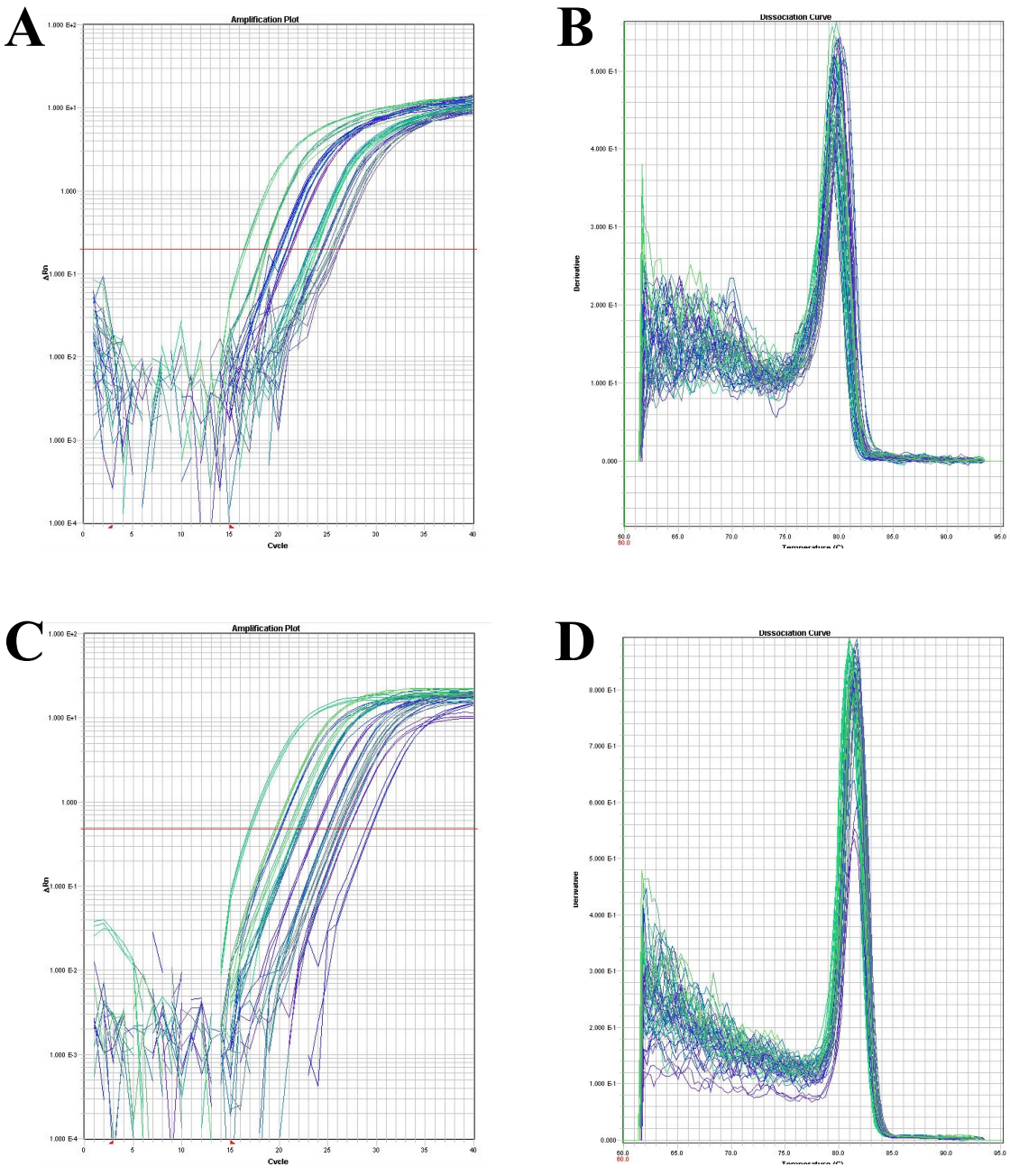
quantify concentrations of the GSTA3 mRNA in each of the tissues. This was accomplished through the use of specific qRT-PCR primers designed to generate short amplicons of 91 bases. Primers were designed with PrimerExpress (ABI) software version 3.0 and ordered from IDT. Two sets of qRT-PCR primers were used: a set of forward and reverse GSTA3 specific primers and a set of forward and reverse GAPDH primers (Table 4). GAPDH mRNA concentrations were measured for possibly normalizing the GSTA3 mRNA concentrations. GAPDH mRNA is commonly used for this purpose and is consistent when used in a single tissue. However, its expression level varies across tissues and is generally higher in metabolically active tissues (Sun et al., 2012). Pseudogenes which are partially expressed have also been identified for GAPDH, which can also cause variation in expression level values obtained from qRT-PCR (Sun et al., 2012). Investigators have evaluated possible primer designs which would not bind GAPDH pseudogene cDNA and have had no success (Sun et al., 2012).

Table 4
Primer sequences used in real time quantitative PCR

Gene Abbr.	Sense	Antisense
GSTA3 - Eca	ACATCCACCTGGTGGAACTTCTCTACCTT	CTGGTTTTCAGGGCCTTCAG
GSTA3 - Cfa	ACATCCACCTGGTTGAACTTCTCTACT	CTGGTTTTCAGGGCCTTCAG
GAPDH - Eca	AAGGTCGGAGTAAACGGATTTG	CCACTTTGCCAGAGTTAAAAGCA
GAPDH - Cfa	AAGGTCGGAGTAAACGGATTTG	CCACTTTGCCAGAGTTAAAAGCA

To perform the qRT-PCR reaction, two master mixes were made, one for each set of primers. The master mixes included the forward and reverse primers, Power SYBR Green PCR Master Mix (Applied Biosystems), and nuclease free H_2O (Life Technologies, Thermo Fisher Scientific, Inc.). The master mixes were pipetted into their designated wells in the 96-well qRT-PCR plate and then cDNA from the reverse transcriptions were added in triplicates to pre-determined wells. The plate was then sealed and placed into the ABI 7900HT Fast Real Time PCR System to run an initial denaturation at 95^{o}C for 15 seconds, then 40 cycles of 95^{o}C denaturation for 15 seconds, followed by 60^{o}C annealing and extension for one minute. This was followed by a 20 minute procedure producing a dissociation curve to assess the melting temperature of the amplicons. Figure 4 displays an example of the amplification and dissociation curves which were obtained. Data resulting from qRT-PCR were then analyzed and utilized in the construction of a table displaying the GSTA3 concentration relative to each tissue for the species used.

Figure 4 Example amplification plots and dissociation curves from qRT-PCR of horse (*Equus caballus*) and dog (*Canis lupus familiaris*) GSTA3 mRNA. Graphs A and B are from horse samples, graph A is the amplification curve and graph B is the dissociation curve. Graphs C and D are from dog, graph C is the amplification curve and graph D is the dissociation curve.



Data Analysis

Each species yielded mRNA sequences which were then analyzed using multiple sequence comparison programs as well as analyzed by hand by comparison of the printed sequence to the chromatogram to check for any obvious read errors. Sequences for each species were sorted based on length and quality of the chromatogram and primers were located within the sequence. They were then analyzed with the NCBI BLAST nucleotide algorithm to determine their orientation and identity to other known sequences. Those sequences bearing high identity to known glutathione S-transferase alpha sequences were then further examined in NCBI BLAST by the use of a multiple sequence alignment evaluation of their identity with the reference GSTA3 mRNA sequence (NM_000847.1) and the horse GSTA3 previously cloned (KC52384) (Ing et al., 2014).

The final sequences chosen for each species were then loaded into the Clustal Omega multiple sequence alignment program along with both the human and horse reference sequences being utilized in this project. GSTA3 mRNA sequences for each species were translated into their amino acid sequences through the use of ExPASy translate tool and then compared using the Clustal Omega multiple sequence alignment program. A GSTA3 mRNA sequence for each species was then submitted to NCBI GenBank and incorporated into the NCBI database.

The data from the multiple tissues qRT-PCR for specific tissue concentrations were analyzed with relative quantification by looking at the amplification plot, the cycle threshold (C_t) values. The dissociation curve graphs were examined for homogeneity of amplicons from individual gene products. C_t values were averaged between replicates, and the ΔCt and $2^{-\Delta\Delta Ct}$ was also calculated for comparison of mRNA concentrations. The data were evaluated with and without normalization to GAPDH mRNA. The data were placed into a table arranged by mRNA concentration values (highest to lowest). Values obtained with greater than two fold difference were considered significantly different while values with less than two fold difference were considered not significantly different and were grouped together. The value for the lowest GSTA3 mRNA expressing tissue was set at one, with other tissue values relative to that. A separate table was constructed for each of the two species analyzed.

CHAPTER IV

RESULTS

RNA Concentration and Purity

Data from the analysis of RNA concentration and purity by Nanodrop spectrophotometer demonstrated that each sample was of acceptable purity. An equal volume of each tissue was used for extraction. All of the samples from testis were of similar concentration except for the chicken which had almost double the concentration of RNA as the others with 3536.2 ng/µl. RNA concentrations were high, thus indicative of plenty of material to work with.

Cloned GSTA3 mRNA Sequences

Due to high coding sequence identity between the members of the GSTA family, a high level of coding sequence identity was required in order to distinguish between members of the family. However, because of the degeneracy of the genetic code, the mRNA sequence did not need to be perfectly identical. From each species, the mRNA sequence which demonstrated the highest identity to the sequences used as reference was chosen, and all sequences including the reference sequences were aligned using the Clustal Omega Multiple Sequence Alignment tool. Output from this alignment is displayed in Figure 5. Percent identity of the GSTA3 mRNAs was determined for each species to each of the reference sequences (human NM_000847.4, and horse

KC512384), which are 85% identical to each other. The GSTA3 mRNA sequences from dog, goat, opossum and chicken testes displayed 88%, 84%, 70% and 71% identity with human GSTA3 mRNA and 87%, 85%, 70% and 69% identity with horse GSTA3 mRNA respectively. These values are arranged in Table 5 for easy comparison. Coding sequences for GSTA3 from each species were deposited in NCBI GenBank. The accession numbers are listed in Table 6.

Table 5
GSTA3 mRNA percent identities

Human	85%	88%	84%	70%	71%
Horse		87%	85%	70%	69%
	Horse	**Dog**	**Goat**	**Opossum**	**Chicken**

Table 6
NCBI accession number for cloned GSTA3 cDNAs

Species	**Accession Number**
Canis lupus familiaris	KJ651954
Capra hircus	KM578828
Gallus gallus	KP686393
Monodelphis domestica	KM977823

Figure 5 Aligned nucleotide sequences of the coding sequences of GSTA3 mRNAs from selected species including those used as reference. Human (*Homo sapiens;* NM_000847.4), horse (*Equus caballus;* KC512384.1), dog (*Canis lupus familiaris;* KJ651954), goat (*Capra hircus;* KM578828), opossum (*Monodelphis domestica;* KM977823), chicken (*Gallus gallus;* KP686393) are those displayed here. Dog, goat, opossum and chicken cDNA sequences were cloned in the current study. Shared identities with the human reference sequence were highlighted in gray. Start and stop codons are identified in bold, green and red type, respectively, and codons for the amino acids critical to the GSTA3 enzyme's isomerase activity have been identified in bold, multicolored type.

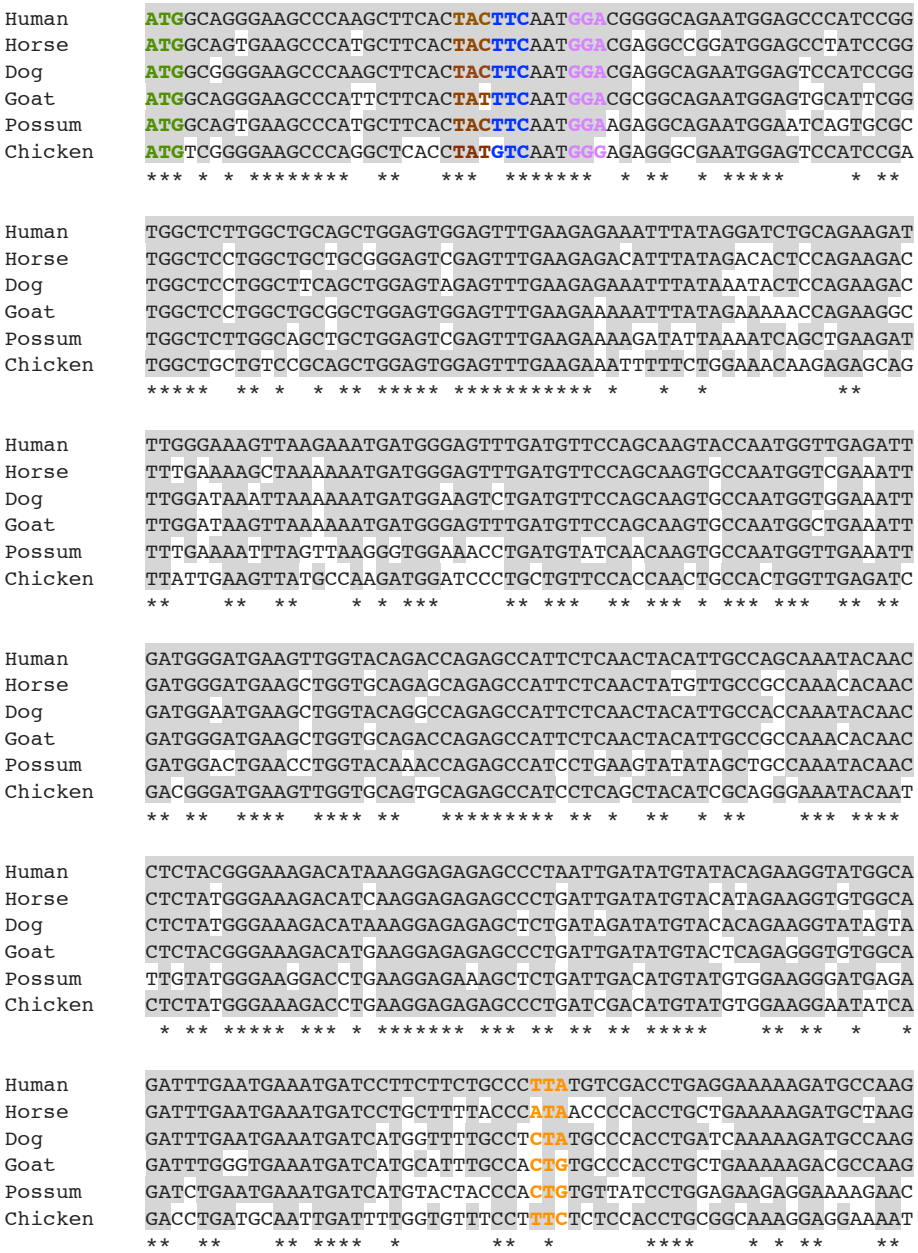
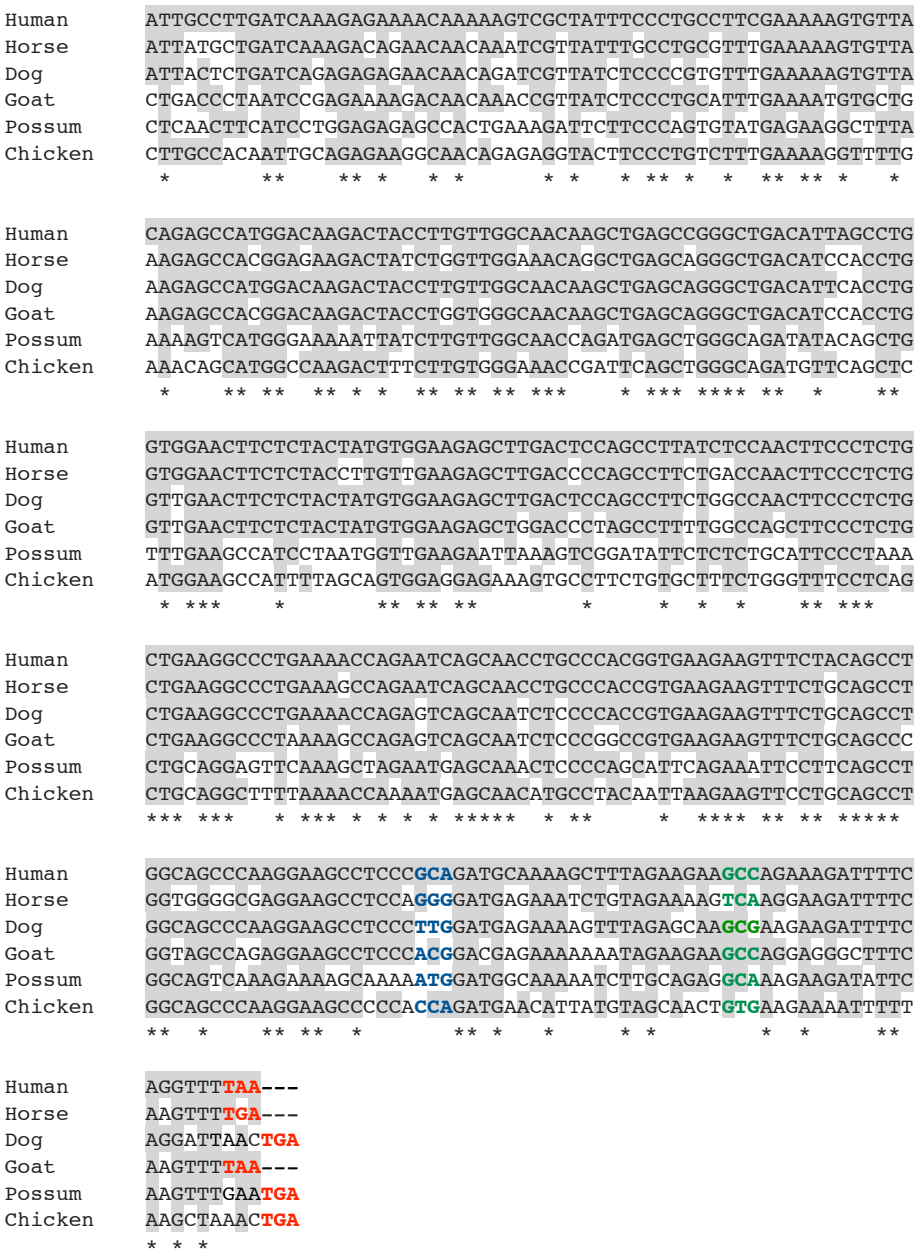


Figure 5 Continued



Cloned GSTA3 protein sequences demonstrated an extremely high degree of identity. The six critical amino acids for 3-ketosteroid isomerase activity of GSTA3 as identified by Dourado et al. (2014), Johansson and Mannervik (2002), and Petterson et al. (2002) which were displayed in Table 1, were located in each sequence. The protein alignment of GSTA3 protein displayed in Figure 6 has the six critical amino acids for 3-ketosteroid isomerase activity highlighted. Percent positives were calculated instead of percent identities for each of the cloned GSTA3 protein sequences to the sequences used as reference. This method was used in order to account for conservative substitutions due to similar chemical properties of an amino acid residue. Results from these calculations displayed 92%, 88%, 77% and 77% positive identity with human GSTA3 protein and 90%, 90%, 77% and 78% positive association with horse GSTA3 protein for the dog, goat, chicken and opossum respectively. The reference human and horse GSTA3 protein sequences demonstrated 90% positive identities when compared to each other. These values are arranged in Table 7. No significant dissimilarities were observed in the locations of the critical amino acids for the GSTA3 enzyme's 3-ketosteroid isomerase activity.

Table 7
GSTA3 protein percent positive identities

Human	90%	92%	88%	77%	77%
Horse		90%	90%	77%	78%
	Horse	**Dog**	**Goat**	**Opossum**	**Chicken**

Figure 6 Aligned amino acid sequences for GSTA3 of all species investigated including those used as reference. The critical amino acids for 3-ketosteroid isomerase activity are highlighted. Symbols: *= identical residues, : = similar residues, . = conservation of groups with weakly similar properties

```
Human         MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEI
Horse         MAVKPMLHYFNGRGRMEPIRWLLAAAGVEFEETFIDTPEDFEKLKNDGSLMFQQVPMVEI
Dog           MAGKPKLHYFNGRGRMESIRWLLASAGVEFEEKFINTPEDLDKLKNDGSLMFQQVPMVEI
Goat          MAGKPILHYFNGRGRMECIRWLLAAAGVEFEEKFIEKPEGLDKLKNDGSLMFQQVPMAEI
Opossum       MAVKPMLHYFNGRGRMESVRWLLAAAGVEFEEKILKSAEDFENLVKGGNLMYQQVPMVEI
Chicken       MSGKPRLTYVNGRGRMESIRWLLSAAGVEFEEIFLETREQLLKLCQDGSLLFHQLPLVEI
              *: ** * *.******* :****::******* :: . * : :* : *.*:::*:*:.**

Human         DGMKLVQTRAILNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPLCRPEEKDAK
Horse         DGMKLVQSRAILNYVAAKHNLYGKDIKERALIDMYIEGVADLNEMILLLPITPPAEKDAK
Dog           DGMKLVQARAILNYIATKYNLYGKDIKERALIDMYTEGIVDLNEMIMVLPLCPPDQKDAK
Goat          DGMKLVQTRAILNYIAAKHNLYGKDMKERALIDMYSEGVADLGEMIMHLPLCPPAEKDAK
Opossum       DGLNLVQTRAILKYIAAKYNLYGKDLKEKALIDMYVEGMRDLNEMIMYYPLCYPGEEEKN
Chicken       DGMKLVQCRAILSYIAGKYNLYGKDLKERALIDMYVEGISDLMQLILVFPFSPPAAKEEN
              **::*** ****.*:* *:******:**:****** **: ** ::*:  *:  *  :: :

Human         IALIKEKTKSRYFPAFEKVLQSHGQDYLVGNKLSRADISLVELLYYVEELDSSLISNFPL
Horse         IMLIKDRTTNRYLPAFEKVLKSHGEDYLVGNRLSRADIHLVELLYLVEELDPSLLTNFPL
Dog           ITLIRERTTDRYLPVFEKVLKSHGQDYLVGNKLSRADIHLVELLYYVEELDSSLLANFPL
Goat          LTLIREKTTNRYLPAFENVLKSHGQDYLVGNKLSRADIHLVELLYYVEELDPSLLASFPL
Opossum       LNFILERATERFFPVYEKALKSHGKNYLVGNQMSWADIQLFEAILMVEELKSDILSAFPK
Chicken       LATIAEKATERYFPVFEKVLKQHGQDFLVGNRFSWADVQLMEAILAVEEKVPSVLSGFPQ
              :  * ::::..*::*.:*:.*:.**:::*****::* **: *.* :  ***   .::: **

Human         LKALKTRISNLPTVKKFLQPGSPRKPPADAKALEEARKIFRF-
Horse         LKALKARISNLPTVKKFLQPGGARKPPGDEKSVEKSRKIFKF-
Dog           LKALKTRVSNLPTVKKFLQPGSPRKPPLDEKSLEQAKKIFRIN
Goat          LKALKARVSNLPAVKKFLQPGSQRKPPTDEKKIEEARRAFKF-
Opossum       LQEFKARMSKLPSIQKFLQPGSQRKAKMDGKNLAEAKKIFKFE
Chicken       LQAFKTKMSNMPTIKKFLQPGSPRKPPPDEHYVATVKKIFKLN
              *: :*::::*::*:::******. **   * : :   :: *::
```

Multiple Tissues GSTA3 mRNA Concentrations

Triplicates of each tissue sample were run for both GAPDH and GSTA3. Calculations for relative quantification ($2^{-\Delta\Delta Ct}$) were completed. The normalized and non-normalized GSTA3 mRNA values of the lowest expressing tissue were then set to one with other tissue values relative to that. Tissues were ranked according to GSTA3 concentration. These values were then utilized to construct tables to easily demonstrate which tissues had a higher concentration of the GSTA3 gene. Tissues with more than a four-fold change in concentration were separated by a V to allow quick visual distinction and the tissue with the highest concentration of GSTA3 is listed at the top. Data for GSTA3 mRNA expression levels are displayed below in Tables 8 and 9 and the GSTA3 mRNA expression levels normalized to GAPDH mRNA expression can be found in Appendix D, Tables A-1 and A-2. Normalized data were placed in the appendix due to the variability of GAPDH mRNA expression across tissues and the possible effects of this on the normalized GSTA3 mRNA expression values.

The samples from *Equus caballus* which demonstrated the greatest GSTA3 mRNA concentrations were testes and the adrenal glands. These results were as expected. Much of the literature describes the highest expression of the GSTA3 gene as localized in steroidogenic tissues. Samples from horse liver demonstrated intermediate levels of GSTA3 mRNA expression. Tissues with extremely low concentrations included the cerebrum, skeletal muscle, heart and mammary. Some samples from this experiment were sequenced and verified as GSTA3 mRNA.

GSTA3 mRNA levels were analyzed in *Canis lupus familiaris* tissue samples. Dog cerebrum, skeletal muscle, heart, mammary gland, uterus kidney and hypothalamus had the lowest concentrations of GSTA3 mRNAs. Although the testes demonstrated high concentrations of the GSTA3 gene, the liver demonstrated the highest concentrations, with concentrations in the adrenal glands and small intestine slightly lower. Due to the role of the GSTAs as detoxification enzymes this finding was not surprising. GSTA3's main role is as a steroid isomerase, but it still retains its detoxification activity. Other studies have observed high expression levels of GSTA3 mRNA in these tissues as well (Gropp et al., 2006). It is also possible that the primers used did not selectively bind GSTA3 mRNA and that GSTA1 mRNA expression levels is represented in the data as well. The sense primer used had two mismatches with the GSTA1 mRNA sequence and the antisense primer had one mismatch. One mismatch from each primer was located at the 3' end which should have allowed for selective binding. These mismatches are displayed in Figure 7. The dogs from which the tissues were obtained were research animals and were fairly old (7 to 12 years of age). Continued investigation is warranted to provide further support for this data.

Table 8

GSTA3 mRNA levels in equine tissues measured by real time quantitative polymerase chain reaction. The lowest value was set to one with all other values relative to that in order to rank the tissues based on their concentrations. "V" designates a 4 fold or more difference between tissue values. A table displaying the ranking after normalization to GAPDH mRNA expression is not shown due to the high variability of GAPDH mRNA expression across tissues but has been included in Appendix D, Table D-1.

Testes (1130), Adrenal (1000)
V
Liver (250)
V
Small Intestine (90), Ovary (70), Kidney Cortex (50), Hypothalamus (40)
V
Uterus (9), Urinary Bladder, Spleen (7), Lung, Skeletal Muscle (4)
V
Heart, Mammary (2), Cerebrum (1)

Table 9

GSTA3 mRNA levels in canine tissues measured by real time quantitative polymerase chain reaction. The lowest value was set to one with all other values relative to that in order to rank the tissues based on their concentrations. "V" designates a 4 fold or more difference between tissue values. A table displaying the ranking after normalization to GAPDH mRNA expression is not shown due to the high variability of GAPDH mRNA expression across tissues but has been included in Appendix D, Table D-2.

Liver (4762)
V
Testes (754), Small Intestine (556)
V
Adrenal (281), Ovary 1 (156.5), Skeletal Muscle (143), Ovary with Follicle (137)
V
Spleen (41)
V
Heart (18), Ovary 2 (17)
V
Mammary (9), Cerebrum (8)
Kidney (6), Hypothalamus (4)
V
Uterus (1)

Figure 7 Double stranded DNA sequences of the putative amplicons of GSTA1 and GSTA3 with primer binding sites for primers used in qRT-PCR highlighted to demonstrate the primers' specificity for GSTA3. Asterisks denote an exact match of the primer to the DNA sequence.

Horse GSTA1 (NM_001284532) with GSTA3 qRT-PCR primers:

```
*************************  *
ACATCCACCTGGTGGAACTTCTCTACTATGTCGAAGAGGTTGACCCCAGCCTTCTGGCCA
TGTAGGTGGACCACCTTGCCGCGCTGATACAGCTTCTCCAACTGGGGTCGGAAGACCGGT

ACTTCCCTCTGCTGAAGGCCCTAAAAACCAG
TGAAGGGAGACGACTTCCGGGATTTTTGGTC
           *********** ********
```

Horse GSTA3 (KC512384) with GSTA3 qRT-PCR primers:

```
*****************************
ACATCCACCTGGTGGAACTTCTCTACCTTGTTGAAGAGCTTGACCCCAGCCTTCTGACCA
TGTAGGTGGACCACCTTGAAGAGATGGAACAACTTCTCGAACTGGGGTCGGAAGACTGGT

ACTTCCCTCTGCTGAAGGCCCTGAAAGCCAG
TGAAGGGAGACGACTTCCGGGACTTTCGGTC
           *************** ****
```

Dog GSTA1 (DN337690.1) with GSTA3 qRT-PCR primers:

```
****  ***************  ******
ACATTCACCTGGTTGAACTTTTCTACTATGTGGAAGAGCTTGACTCCAGCCTTTTGGCCA
TGTAAGTGGACCAACTTGAAAAGATGATACACCTTCTCGAACTGAGGTCGGAAAACCGGT

ACTTTCCTCTGGTGAAGGCCCTGAAAACCAG
TGAAAGGAGACCACTTCCGGGACTTTTGGTC
             ********************
```

Dog GSTA3 (KJ651954) with GSTA3 qRT-PCR primers:

```
****  ***********************
ACATTCACCTGGTTGAACTTCTCTACTATGTGGAAGAGCTTGACTCCAGCCTTCTGGCCA
TGTAAGTGGACCAACTTGAAGAGATGATACACCTTCTCGAACTGAGGTCGGAAGACCGGT

ACTTCCCTCTGCTGAAGGCCCTGAAAACCAG
TGAAGGGAGACGACTTCCGGGACTTTTGGTC
           ********************
```

CHAPTER V

DISCUSSION

The coding sequence of the Glutathione S-transferase alpha 3 mRNA is well conserved across species including those of a divergent nature. The highest observed concentrations are localized to tissues involved in steroidogenesis and detoxification as was expected based on previously published literature and the known chemical functions of the GSTA family of enzymes. These results provide further support for the research demonstrating the greater activity of the GSTA3 enzyme to that of the HSD3B2 enzyme as a 3-ketosteroid isomerase in the △5 testosterone production pathway and to the need for precisely chosen research animals when modeling a specific system or pathway. Also, this knowledge provides building blocks to fully elucidating the mechanisms behind steroidogenesis which will provide the ability for important advances in the medical research relating to numerous diseases and treatments. However, theres still a need for further elucidation of the mechanisms of regulation behind the actions of the GSTA3 enzyme.

Steroidogenesis

Matsumura and colleagues (2013) have taken on the question of regulation of the GSTA3 gene and have demonstrated regulation of the GSTA family by the actions of steroidogenic factor 1 (SF-1) which is a nuclear orphan receptor and a master regulator

for steroidogenesis (Parker and Schimmer, 1997). This study revealed through the use of a chromatin immunoprecipitation assay (ChIP) that SF-1 binds directly to the promoter of the GSTA3 gene and thus up regulates transcription (Matsumura et al., 2013). SF-1 has also been linked to tissue specific expression of genes involved in steroid hormone biosynthesis and, when knocked out in mice, failure of adrenal and gonadal development and impaired gonadotrope function were observed (Parker and Schimmer, 1997). Normal development of the reproductive system and sex hormone production is required for fertility. A clear link between reduced fertility/sex hormone production levels and stress has also been defined by other researchers. Many studies have utilized glucocorticoids to research the physiological effects of stress, and have documented glucocorticoid concentration changes during different events ranging from trailering of horses (Schmidt et al., 2010), scrotal insulation and dexamethasone treatment in bulls (Barth and Bowman, 1994) to aging and obesity in humans (Blouin et al., 2006; Chung et al., 2001; Wang and Stocco, 2005), as well as a number of other conditions (Cooke et al., 1991; Cornelisse et al., 2004; Danek, 2004; Gold et al., 2012; Grady et al., 2010; Haffner et al., 2009; Hardy et al., 2005; Ing et al., 2014, 2015; Kizaki et al., 1998; Liu et al., 2011; Lopez-Calderon et al., 1991; Quax et al., 2013).

Kizaki and colleagues (1998) demonstrated significant age related increases in glucocorticoid production in mice. They hypothesized that this is a consequence of progressive dysfunction of the hypothalmic-pituitary-adrenal axis. As these changes occur there is a corresponding decrease in testosterone production which contributes to

the hypothesis that glucocorticoids have a role in regulating steroidogenesis. This hypothesis is supported by the presence of glucocorticoid receptors in the Leydig cells as well as the demonstration of apoptosis in Leydig cells in rats and horses after corticosterone administration (Payne and Youngblood, 1995; Yoon and Roser, 2010). The Leydig cell is the most efficient cell in the male at using cholesterol to synthesize testosterone and sufficient numbers of functional Leydig cells is required to maintain the necessary levels of testosterone production (Payne and Youngblood, 1995). Adequate testosterone production is required for normal male sexual differentiation and fetal development as well as the initiation and maintenance of spermatogenesis and expression of male secondary sex characteristics (Payne and Youngblood, 1995).

Age related changes have been documented in relation to Sertoli cell, Leydig cell, testosterone concentration and sperm production. In the horse specifically, puberty begins at one to one and a half years of age, and adult numbers of Leydig cell, Sertoli cell and sperm production are reached by age four (Sipahutar et al., 2003). The stallion demonstrates heterogeneity in composition due to light and dark Leydig cell populations, a characteristic shared with the rat which demonstrates a commonality between mammalian species (Sipahutar et al., 2003) This is hypothesized to be related to different steroidogenic capacities in the two Leydig cell types (Sipahutar et al., 2003). Leydig cell aging is a popular region of study due to its immense impact on hormone balance and thus on long term health and disease risk factors. The ability to induce a steroidogenic dormant period by which aging of Leydig cells is halted was demonstrated

by Chen and Zirkin (1999). In this study rats were given Silastic implants which delivered sustained release testosterone and halted endogenous production. After eight months the implants were removed and two months later testosterone production levels were compared between the treatments and controls identifying a significant decrease in control rats (Chen and Zirkin, 1999). The authors postulated a delay in Leydig cell aging and the subsequent reduction in testosterone and its related adverse health consequences as an application of these findings with the use of male contraceptives. The mechanisms by which this outcome occurred and the unobserved effects on the production other components of the testosterone biosynthesis such as HSD3B2 and GSTA3 must first be investigated.

Glutathione S-transferase Alpha 3

In this project the active site residues proposed by Johansson and Mannervik (2002) and Dourado et al. (2014) for glutathione S-transferase alpha 3's isomerase activity were located in the amino acid sequences of multiple species including divergent species *Monodelphis domestica* and *Gallus gallus.* This demonstrates the conservation of the enzyme in species which utilize the $\triangle 5$ steroidogenic pathway. In 2002 Johansson and Mannervik reported a mechanism for the isomerase activity. More recently, Calvaresi and colleagues (2012) performed an investigation into the catalytic activity of the GSTA3 enzyme. This team of researchers utilized Quantum Mechanics/ Molecular Mechanics to computationally elucidate the catalytic mechanisms behind the

isomerase activity of human GSTA3. Their research not only validated Johansson and Mannervik's (2002) hypothesis that the GSH cofactor acts as an acid/base catalyst, but also provided a detailed description of the mechanism behind the isomerization reaction. The reaction was originally thought to be a two-step process but was shown to be concerted, highly asynchronous and fully dependent on the contributions of the active site residues outlined by other authors (Calvaresi et al, 2012; Dourado et al., 2014; Johannson and Mannervik, 2002; Petterson et al., 2002). These findings are corroborative to the mRNA sequences of GSTA3 obtained in this study and its importance in the △5 testosterone biosynthesis pathway.

There are many species which utilize the △5 testosterone biosynthesis pathway in which the presence of GSTA3 is still unverified. Elucidation of specific species which employ this enzyme is intrinsic to fully comprehending the mechanisms behind testosterone biosynthesis and reproductive function for species like humans and livestock in which research is not always an option. The mouse is the current most popular organism for this purpose, however, rodents utilize the △4 pathway of steroidogenesis. For a complete understanding of the △5 testosterone biosynthesis pathway and maximum application of this knowledge, steroidogenesis should be investigated in many species, including those of a divergent nature.

Continued research into GSTA3 gene products and the effects of glucocorticoids will further demonstrate the regulatory mechanisms which alter testosterone

concentrations and thus impact fertility and possibly over-all health of an individual. Due to the ever increasing genetic information the identification of species which utilize GSTA3 and the $\triangle$5 testosterone production pathway should be an easier task than it was previously. The availability of completed genomes in databases like NCBI will allow for many species to be identified before confirming the genes presence experimentally, thus saving time and money. Execution of a large cross-species comparison study on the regulation of the GSTA3 gene would yield valuable data with high applicability to the fields of reproductive physiology and medicine.

CHAPTER VI

CONCLUSION

The glutathione S-transferase alpha 3 mRNA sequence is well conserved across species which utilize the △5 testosterone biosynthesis pathway and is most highly expressed in steroidogenic tissues. This study has demonstrated this conservation occurs not only in closely related species but also across divergent species. Conservation of this gene across such a broad range of species is indicative of its importance in steroidogenesis. It is crucial that a complete understanding of steroidogenesis is developed considering the numerous steroid hormone-dependent diseases and endocrine disorders need novel treatments. As a recently discovered enzyme, GSTA3 requires extensive analysis to fully identify its mechanisms of action in the △5 testosterone biosynthesis pathway.

Expression of the pET-21a clones and evaluation of their steroid isomerase activity will also provide useful information for designing future experiments. The pET-21a expression vector utilizes bacteriophage T7 transcription and expression is induced by T7 RNA polymerase (Novagen, 2003). When fully induced, T7 RNA polymerase can convert most of the cell's resources to target gene expression and within a few hours the desired product can constitute more than 50% of the total cell protein (Novagen, 2003). The pET-21a expression vector also contains a multiple cloning

region which contains unique restriction sites in order to utilize directional cloning of the target sequence (Novagen, 2003). This will increase the number of clones capable of expression. Proteins expressed by the pET-21a vector do not contain vector encoded sequences (Novagen, 2003). The construction of this system allows for the efficient production of large amounts of native protein and quick purification.

Expression and analysis of the GSTA3-pET-21a clones generated during this experiment will produce quantitative comparisons of the steroid isomerase activity of the GSTA3 enzyme between the species investigated. Many regulators of the GSTA3 gene have been identified. The availability of expressed GSTA3-pET-21a clones will allow for further investigation of the effects of endogenous compounds like FSH, testosterone, estradiol and glucocorticoids, as well as pharmaceuticals like phenobarbital and dexamethasone on the expression of the GSTA3 enzyme. Insights into those compounds which regulate the GSTA3 enzyme can impart medically relevant advances in treating androgen and estrogen dependent diseases. Considering the value and distribution of pharmaceuticals utilized in the treatment of diseases like prostate cancer, breast cancer, polycystic ovary syndrome, and infertility, it is essential that the treatments that are prescribed are both specific and effective.

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APPENDIX A

Human GSTA mRNA Alignment

Figure A-1 Aligned nucleotide sequences of the coding sequences of GSTA mRNAs from humans only. Human (*Homo sapiens*) GSTA3 (NM_000847.4), GSTA1 (NM_145740.3), GSTA2 (NM_000846.4), and GSTA5 (NM_153699.1) with highlighted regions of identity to GSTA3.

```
hGSTA3     ATGGCAGGGAAGCCCAAGCTTCACTACTTCAATGGACGGGGCAGAATGGAGCCCATCCGG
hGSTA1     ATGGCAGAGAAGCCCAAGCTCCACTACTTCAATGCACGGGGCAGAATGGAGTCCACCGG
hGSTA2     ATGGCAGAGAAGCCCAAGCTCCACTACTCCAATATACGGGGCAGAATGGAGTCCATCCGG
hGSTA5     ATGGCAGAGAAGCCCAAGCTCCACTACTCCAATGCACGGGGCAGTATGGAGTCCATTCGG
           ******* ************ ******* ****  ********* ****** ***  ***

hGSTA3     TGGCTCTTGGCTGCAGCTGGAGTGGAGTTTGAAGAGAAATTTATAGGATCTGCAGAAGAT
hGSTA1     TGGCTCCTGGCTGCAGCTGGAGTAGAGTTTGAAGAGAAATTTATAAAATCTGCAGAAGAT
hGSTA2     TGGCTCCTGGCTGCAGCTGGAGTAGAGTTTGAAGAGAAATTTATAAAATCTGCAGAAGAT
hGSTA5     TGGCTCCTGGCTGCAGCTGGAGTAGAGTTGGAAGAGAAATTTCTAGAATCTGCAGAAGAT
           ****** ***************** ***** ************ **  **************

hGSTA3     TTGGGAAAGTTAAGAAATGATGGGAGTTTGATGTTCCAGCAAGTACCAATGGTTGAGATT
hGSTA1     TTGGACAAGTTAAGAAATGATGGATATTTGATGTTCCAGCAAGTGCCAATGGTTGAGATT
hGSTA2     TTGGACAAGTTAAGAAATGATGGATATTTGATGTTCCAGCAAGTGCCAATGGTTGAGATT
hGSTA5     TTGGACAAGTTAAGAAATGATGGGAGTTTGCTGTTCCAGCAAGTACCAATGGTTGAGATT
           ****  ******************   **** ************ ***************

hGSTA3     GATGGGATGAAGTTGGTACAGACCAGAGCCATTCTCAACTACATTGCCAGCAAATACAAC
hGSTA1     GATGGGATGAAGCTGGTGCAGACCAGAGCCATTCTCAACTACATTGCCAGCAAATACAAC
hGSTA2     GATGGGATGAAGCTGGTGCAGACCAGAGCCATTCTCAACTACATTGCCAGCAAATACAAC
hGSTA5     GACGGGATGAAGCTGGTGCAGACCAGAGCCATTCTTAACTACATTGCCAGCAAATACAAC
           ** ********* **** ***************** ************************

hGSTA3     CTCTACGGGAAAGACATAAAGGAGAGAGCCCTAATTGATATGTATACAGAAGGTATGGCA
hGSTA1     CTCTATGGGAAAGACATAAAGGAGAGAGCCCTGATTGATATGTATATAGAAGGTATAGCA
hGSTA2     CTCTATGGGAAAGACATAAAGGAGAAAGCCCTGATTGATATGTATATAGAAGGTATAGCA
hGSTA5     CTTTATGGGAAAGACATGAAGGAGAGAGCCCTGATTGATATGTACACAGAAGGTATAGTA
           ** ** *********** ******* ****** *********** * ********* * *

hGSTA3     GATTTGAATGAAATGATCCTTCTTCTGCCCTTATGTCGACCTGAGGAAAAAGATGCCAAG
hGSTA1     GATTTGGGTGAAATGATCCTCCTTCTGCCCGTATGTCCACCTGAGGAAAAAGATGCCAAG
hGSTA2     GATTTGGGTGAAATGATCCTTCTTCTGCCCTTTAGTCAACCTGAGGAACAAGATGCCAAG
hGSTA5     GATTTGACTGAAATGATCCTTCTTCTGCTCATATGTCAACCAGAGGAAAGAGATGCCAAG
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Figure A-1 Continued

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hGSTA3     ATTGCCTTGATCAAAGAGAAAACAAAAAGTCGCTATTTCCCTGCCTTCGAAAAAGTGTTA
hGSTA1     CTTGCCTTGATCAAAGAGAAAATAAAAAAATCGCTACTTCCCTGCCTTTGAAAAAGTCTTA
hGSTA2     CTTGCCTTGATCCAAGAGAAAACAAAAAAATCGCTACTTCCCTGCCTTTGAAAAAGTCTTA
hGSTA5     ACTGCCTTGGTCAAAGAGAAAATAAAAAAATCGCTACTTCCCTGCCTTTGAAAAAGTCTTA
             ******* ** ********* ***** ****** ********** ******** ***

hGSTA3     CAGAGCCATGGACAAGACTACCTTGTTGGCAACAAGCTGAGCCGGGCTGACATTAGCCTG
hGSTA1     AAGAGCCATGGACAAGACTACCTTGTTGGCAACAAGCTGAGCCGGGCTGACATTCATCTG
hGSTA2     AAGAGCCACGGACAAGACTACCTTGTTGGCAACAAGCTGAGCCGGGCTGACATTCACCTG
hGSTA5     AAGAGCCACAGACAAGACTACCTTGTTGGCAACAAGCTGAGCTGGGCTGACATTCACCTG
            *******  ********************************** ***********   ***

hGSTA3     GTGGAACTTCTCTACTATGTGGAAGAGCTTGACTCCAGCCTTATCTCCAACTTCCCTCTG
hGSTA1     GTGGAACTTCTCTACTACGTCGAGGAGCTTGACTCCAGTCTTATCTCCAGCTTCCCTCTG
hGSTA2     GTGGAACTTCTCTACTACGTGGAAGAGCTTGACTCTAGCCTTATTTCCAGCTTCCCTCTG
hGSTA5     GTGGAACTTTTCTACTACGTGGAAGAGCTTGACTCGAGTCTTATCTCCAGCTTCCCTCTG
           ********* ******* ** ** ********** ** ***** **** **********

hGSTA3     CTGAAGGCCCTGAAAACCAGAATCAGCAACCTGCCCACGGTGAAGAAGTTTCTACAGCCT
hGSTA1     CTGAAGGCCCTGAAAACCAGAATCAGCAACCTGCCCACAGTGAAGAAGTTTCTACAGCCT
hGSTA2     CTGAAGGCCCTGAAAACCAGAATCAGTAACCTGCCCACAGTGAAGAAGTTTCTACAGCCT
hGSTA5     CTGAAGGCCCTGAAAACCAGAATCAGCAACCTGCCCACGGTGAAGAAGTTTCTGCAGCCT
           ************************** ********** ************** ******

hGSTA3     GGCAGCCCAAGGAAGCCTCCCGCAGATGCAAAAGCTTTAGAAGAAGCCAGAAAGATTTTC
hGSTA1     GGCAGCCCAAGGAAGCCTCCCATGGATGAGAAATCTTTAGAAGAAGCAAGGAAGATTTTC
hGSTA2     GGCAGCCCAAGGAAGCCTCCCATGGATGAGAAATCTTTAGAAGAATCAAGGAAGATTTTC
hGSTA5     GGCAGCCAGAGAAAGCCTCCCATGGATGAGAAATCTTTAGAAGAAGCAAGGAAGATTTTC
           *******  ** *********   ****  *** ********** * ** *********

hGSTA3     AGGTTTTAA
hGSTA1     AGGTTTTAA
hGSTA2     AGGTTTTAA
hGSTA5     AGGTTTTAA
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APPENDIX B

Placental Mammals GSTA3 mRNA Alignment

Figure B-1 Aligned nucleotide sequences of the coding sequences of GSTA3 mRNAs from placental species only including those used as reference. Human (*Homo sapiens;* NM_000847.4), horse (*Equus caballus;* KC512384.1), dog (*Canis lupus familiaris;* KJ651954), and goat (*Capra hircus;* KM578828) are those displayed here. Dog and goat mRNA sequences were cloned. Start and Stop codons are identified in green and red text, respectively. Regions identical to human GSTA3 mRNA have been highlighted.

```
Human       ATGGCAGGGAAGCCCAAGCTTCACTACTTCAATGGACGGGGCAGAATGGAGCCCATCCGG
Horse       ATGGCAGTGAAGCCCATGCTTCACTACTTCAATGGACGAGGCCGGATGGAGCCTATCCGG
Dog         ATGGCGGGGAAGCCCAAGCTTCACTACTTCAATGGACGAGGCAGAATGGAGTCCATCCGG
Goat        ATGGCAGGGAAGCCCATTCTTCACTATTTCAATGGACGCGGCAGAATGGAGTGCATTCGG
            ***** * ********  ******** ********** *** * ******   ** ***

Human       TGGCTCTTGGCTGCAGCTGGAGTGGAGTTTGAAGAGAAATTTATAGGATCTGCAGAAGAT
Horse       TGGCTCCTGGCTGCTGCGGGAGTCGAGTTTGAAGAGACATTTATAGACACTCCAGAAGAC
Dog         TGGCTCCTGGCTTCAGCTGGAGTAGAGTTTGAAGAGAAATTTATAAATACTCCAGAAGAC
Goat        TGGCTCCTGGCTGCGGCTGGAGTGGAGTTTGAAGAAAAATTTATAGAAAAACCAGAAGGC
            ****** ***** * ** ***** ********** * *******       ******

Human       TTGGGAAAGTTAAGAAATGATGGGAGTTTGATGTTCCAGCAAGTACCAATGGTTGAGATT
Horse       TTTGAAAAGCTAAAAAATGATGGGAGTTTGATGTTCCAGCAAGTGCCAATGGTCGAAATT
Dog         TTGGATAAATTAAAAAATGATGGAAGTCTGATGTTCCAGCAAGTGCCAATGGTGGAAATT
Goat        TTGGATAAGTTAAAAAATGATGGGAGTTTGATGTTCCAGCAAGTGCCAATGGCTGAAATT
            ** *  **  *** ********* *** *************** *******  ** ***

Human       GATGGGATGAAGTTGGTACAGACCAGAGCCATTCTCAACTACATTGCCAGCAAATACAAC
Horse       GATGGGATGAAGCTGGTGCAGAGCAGAGCCATTCTCAACTATGTTGCCGCCAAACACAAC
Dog         GATGGAATGAAGCTGGTACAGGCCAGAGCCATTCTCAACTACATTGCCACCAAATACAAC
Goat        GATGGGATGAAGCTGGTGCAGACCAGAGCCATTCTCAACTACATTGCCGCCAAACACAAC
            ***** ****** **** ***  ******************  *****  **** *****

Human       CTCTACGGGAAAGACATAAAGGAGAGAGCCCTAATTGATATGTATACAGAAGGTATGGCA
Horse       CTCTATGGGAAAGACATCAAGGAGAGAGCCCTGATTGATATGTACATAGAAGGTGTGGCA
Dog         CTCTATGGGAAAGACATAAAGGAGAGAGCTCTGATAGATATGTACACAGAAGGTATAGTA
Goat        CTCTACGGGAAAGACATGAAGGAGAGAGCCCTGATTGATATGTACTCAGAGGGTGTGGCA
            ***** ********** ********** ** ** ********   *** *** * * *

Human       GATTTGAATGAAATGATCCTTCTTCTGCCCTTATGTCGACCTGAGGAAAAAGATGCCAAG
Horse       GATTTGAATGAAATGATCCTGCTTTTACCCATAACCCCACCTGCTGAAAAAGATGCTAAG
Dog         GATTTGAATGAAATGATCATGGTTTTGCCTCTATGCCCACCTGATCAAAAAGATGCCAAG
Goat        GATTTGGGTGAAATGATCATGCATTTGCCACTGTGCCCACCTGCTGAAAAAGACGCCAAG
            ******  ********** *   * * **  *    * *****   ******* ** ***
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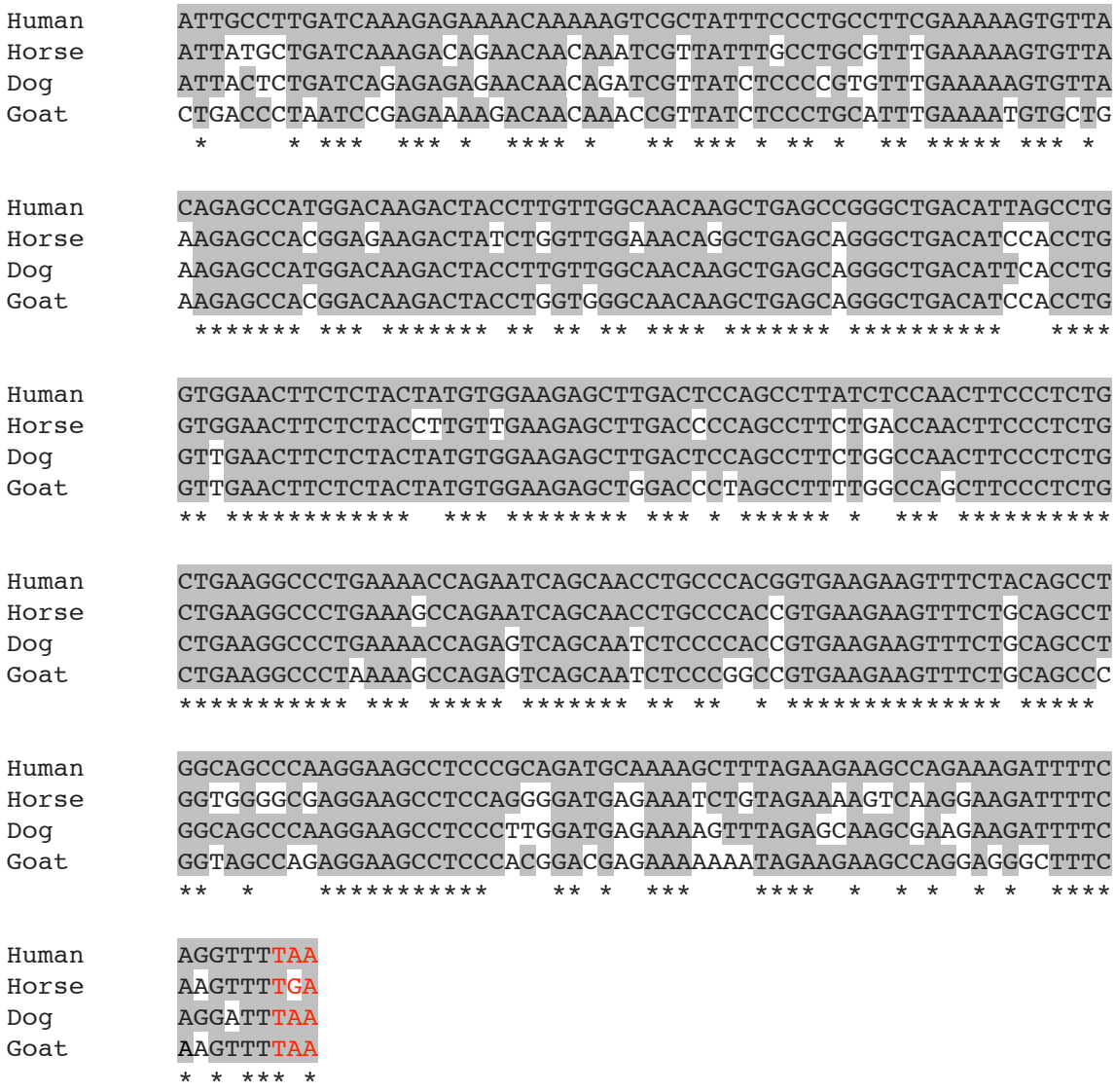
Figure B-1 Continued



APPENDIX C

Plasmid maps of both pCR2.1 and pET-21a

Figure C-1 Plasmid map of dog (*Canis lupus familiaris*) GSTA3-pCR2.1, NCBI accession number KJ651954.

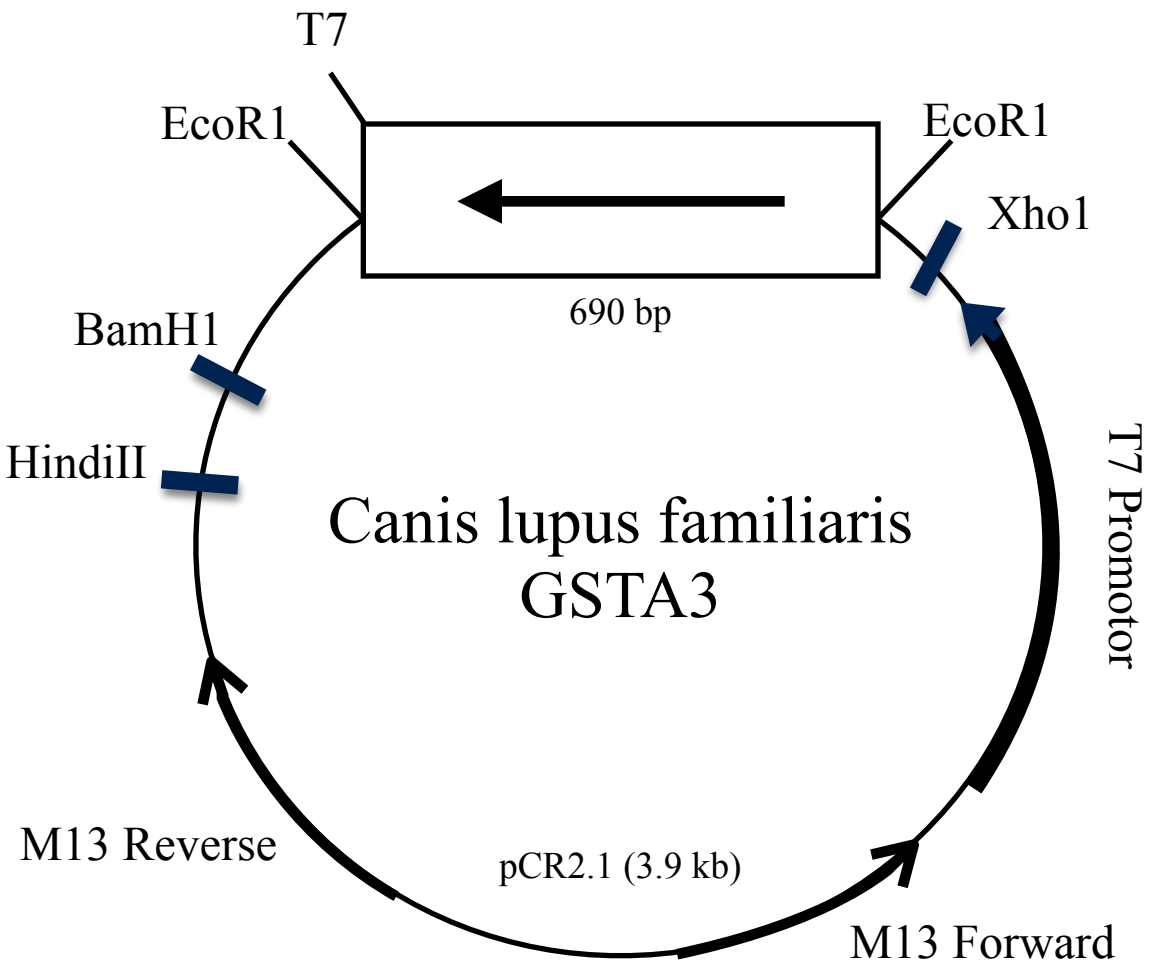


Figure C-2 Plasmid map of goat (*Capra hircus*) GSTA3-pCR 2.1, NCBI accession number KJ746603.

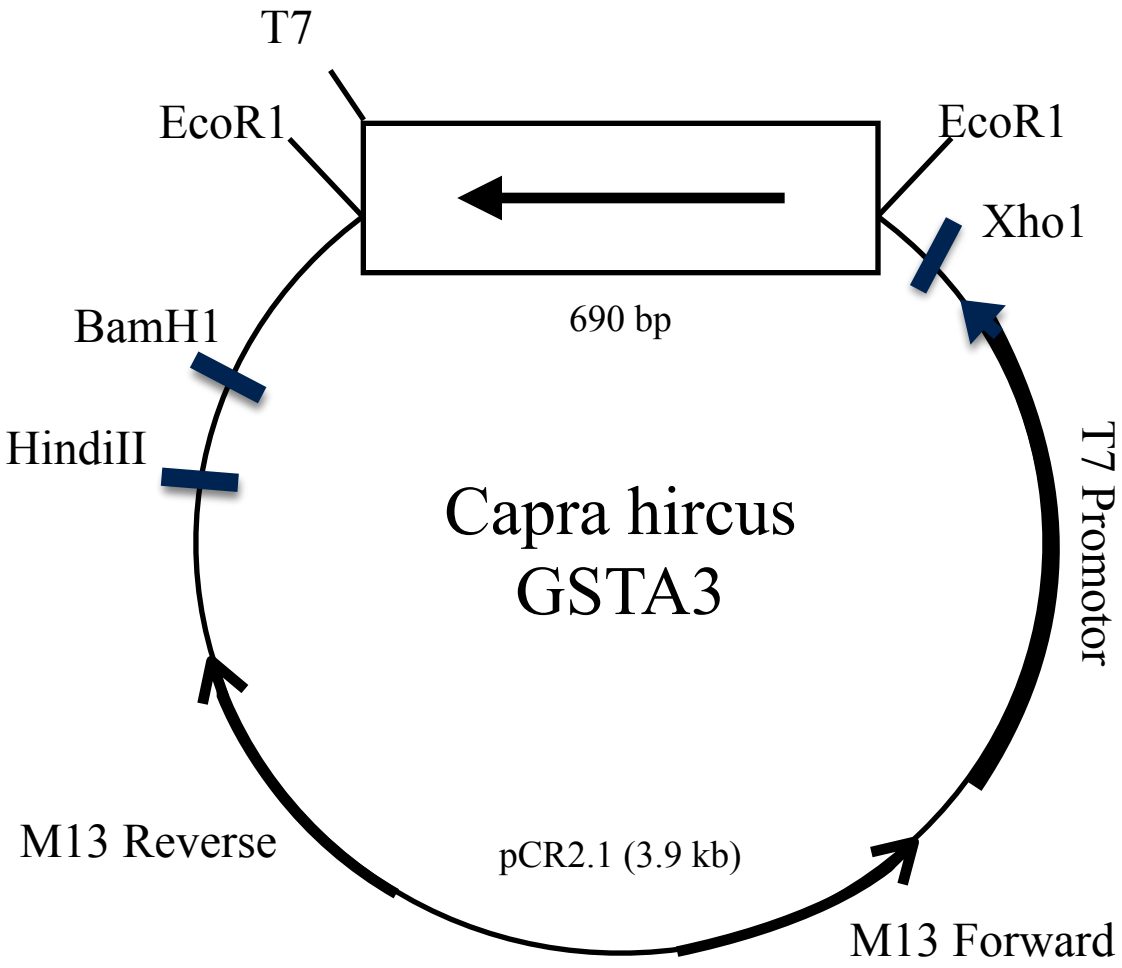


Figure C-3 Plasmid map of opossum (*Monodelphis domestica*) GSTA3-pCR 2.1, NCBI accession number KM977823.

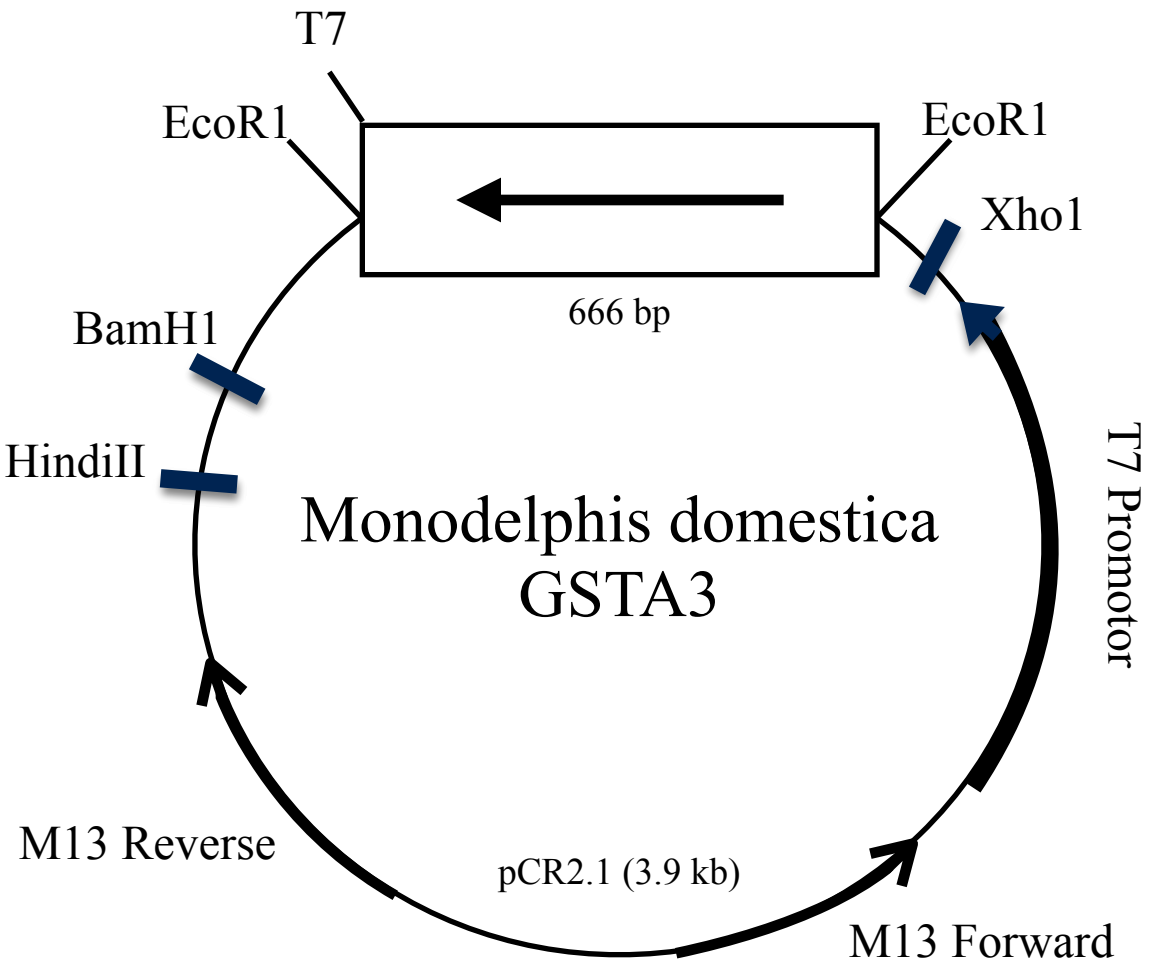


Figure C-4 Plasmid map of dog (*Canis lupus familiaris*) GSTA3-pET-21a, NCBI accession number KJ776127.

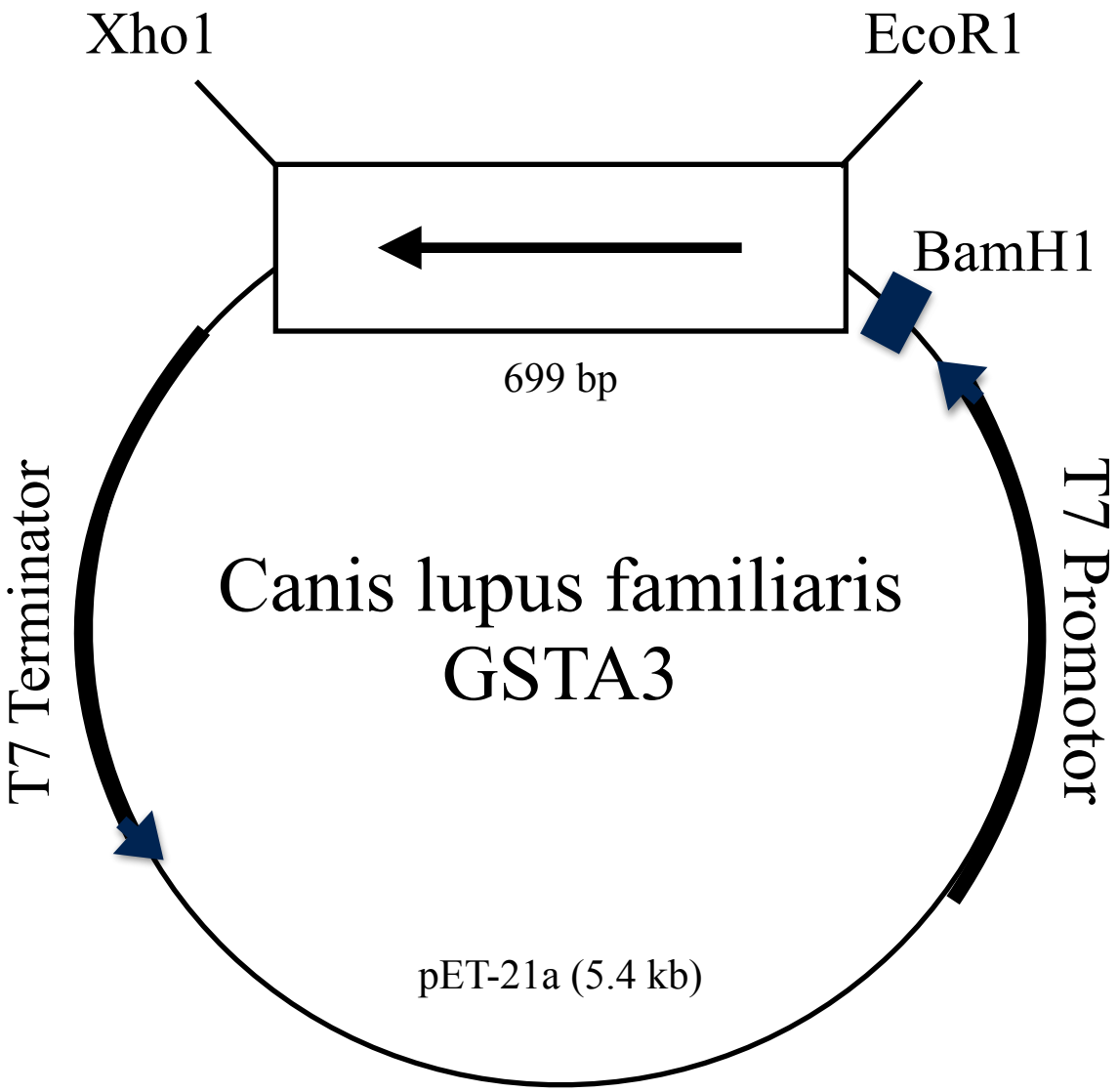


Figure C-5 Plasmid map of goat (*Capra hircus*) GSTA3-pET-21a, NCBI accession number KM578828.

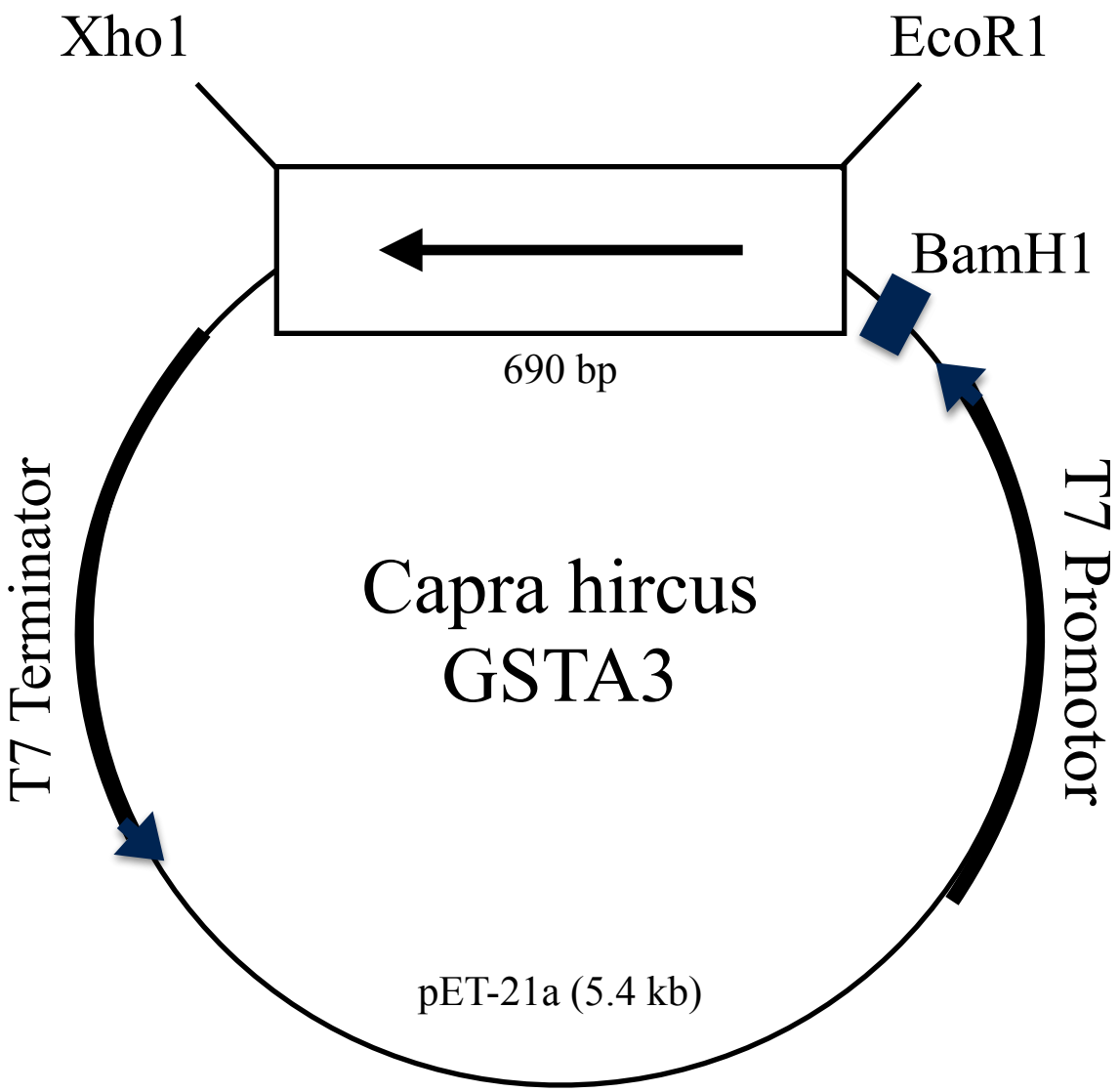


Figure C-6 Plasmid map of opossum (*Monodelphis domestica*) GSTA3-pET-21a, NCBI accession number KP686394.

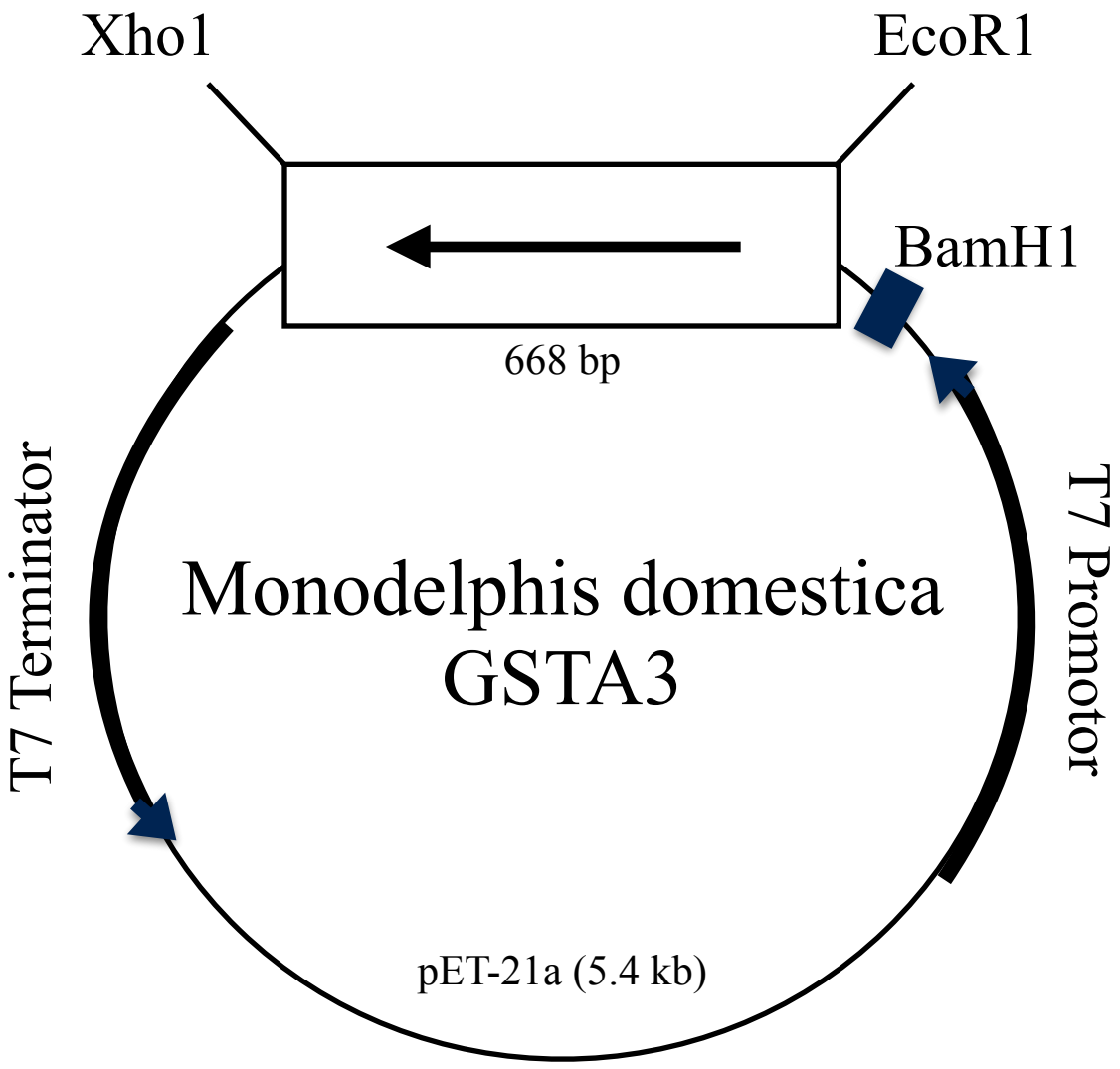
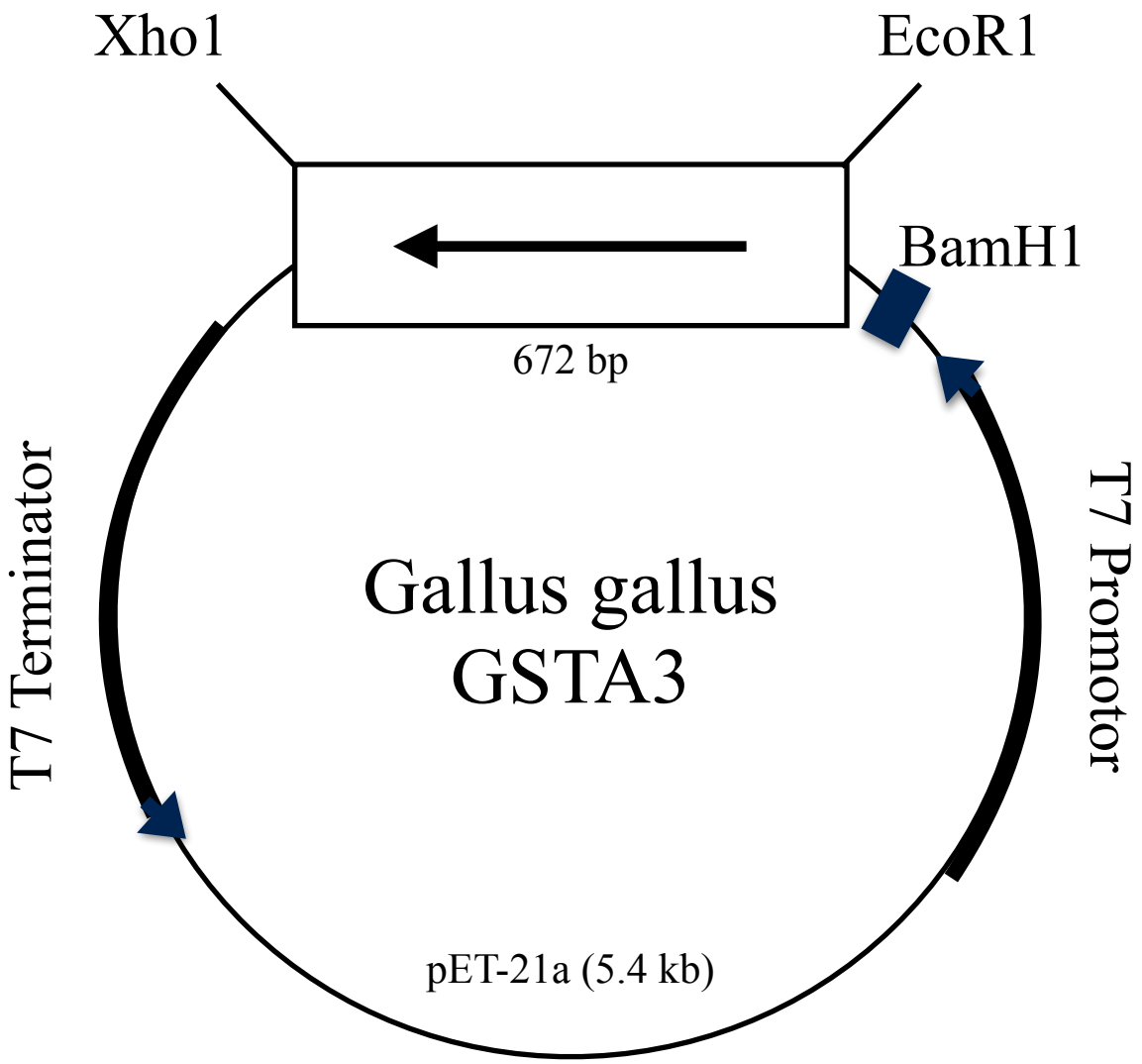


Figure C-7 Plasmid map of chicken (*Gallus gallus*) GSTA3-pET-21a, NCBI accession number KP686393.



APPENDIX D

GSTA3 mRNA expression normalized by GAPDH mRNA expression determined by real time quantitative polymerase chain reaction

Table D-1 GSTA3 mRNA levels in equine tissues normalized to GAPDH mRNA levels measured by real time quantitative polymerase chain reaction. The lowest value was set to one with all other values relative to that in order to rank the tissues based on their concentrations. "V" designates a 4 fold or more difference between tissue values.

Testes (15000)
V
Adrenal (1500), Liver (1000)
V
Ovary (500), Small Intestine (225), Kidney Cortex (150)
V
Hypothalamus (62.5), Uterus (50), Lung, Spleen (25), Urinary Bladder (22.5)
V
Heart (7.5)
V
Mammary (2.5), Cerebrum, Skeletal Muscle (1)

Table D-2 GSTA3 mRNA levels in canine tissues normalized to GAPDH mRNA levels measured by real time quantitative polymerase chain reaction. The lowest value was set to one with all other values relative to that in order to rank the tissues based on their concentrations. "V" designates a 4 fold or more difference between tissue values.

Liver (734)
V
Small Intestine (184.7), Adrenal (132)
Testes (71.8)
Ovary with Follicle (59), Ovary (51.5)
V
Spleen (21)
V
Ovary 1 (10)
V
Skeletal Muscle (4.8), Mammary (3.5), Heart (2.6) Hypothalamus (1.8), Kidney (1.6), Uterus (1.14), Cerebrum (1)